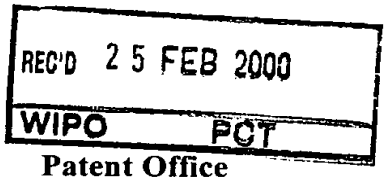
PCT/AU00/00011

#2



Patent Office
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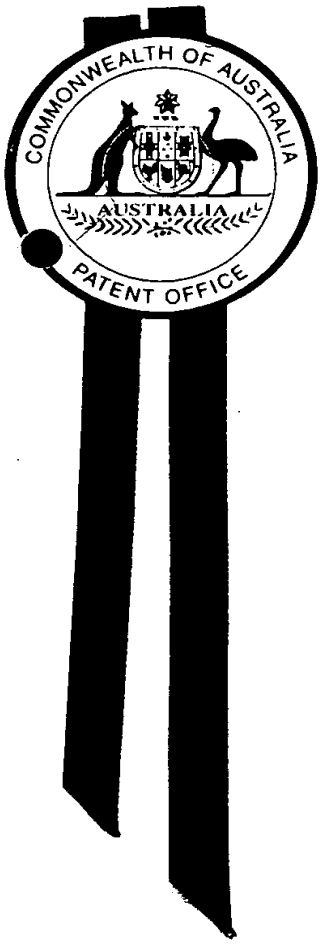

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Twenty-first day of February 2000

LEANNE MYNOTT
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SUPPORT AND SALES

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AUSTRALIA

Patents Act 1990

UNISEARCH LIMITED

PROVISIONAL SPECIFICATION

Invention Title:

Catalytic molecules

The invention is described in the following statement:

Catalytic Molecules

FIELD OF THE INVENTION

The present invention relates to DNAzymes which are targeted against mRNA molecules encoding EGR-1 (also known as Egr-1 or NGFI-A). The present invention also relates to compositions including these DNAzymes and to methods of treatment involving administration of the DNAzymes.

BACKGROUND OF THE INVENTION

Ribozymes are ribonucleic acid (RNA) molecules which have long been recognized for their capacity to selectively bind to an RNA substrate by Watson-Crick base-pairing and cleave phosphodiester bonds (Haseloff & Gerlach, 1988; Saxena & Ackerman, 1990; McCall et al, 1992). This property has been successfully exploited by many groups to inhibit gene expression in a variety of cell types (reviewed in James & Gibson, 1998). However, the utility of ribozymes as biologic and therapeutic tools has been limited by the susceptibility of these molecules to chemical and enzymatic degradation (Simayama et al, 1993; Heidenreich & Eckstein, 1992) and restricted target site specificity. Chimeric ribozymes containing deoxyribonucleic acid (DNA) or phosphorothioate linkages have been generated to overcome sensitivity to degradation, but these ribozymes are expensive to synthesize and prone to degrade in serum. Antisense phosphorothioated oligodeoxynucleotides (ODNs) are more resistant to nucleolytic cleavage, but these molecules lack catalytic activity. A new generation of catalytic nucleic acid composed entirely of DNA has recently been developed using an *in vitro* selection strategy (Santoro & Joyce, 1997). These Mg2+-dependent moieties cleave RNA potentially at any purine-pyrimidine junction (Santoro & Joyce, 1997) and offer greater substrate specificity than hammerhead ribozymes (Kuwabara et al, 1997). Despite the therapeutic promise of DNAzymes, the capacity of these molecules to influence biological responsiveness has not been determined at a cellular or molecular level.

Smooth muscle cells (SMCs) are well recognized as a significant cellular component of atherosclerotic and post-angioplasty restenotic lesions (Stary et al, 1995; Holmes et al, 1984). SMC migration and proliferation are key events in the pathogenesis of these vascular disorders (Jackson & Schwartz, 1992; Libby et al, 1995). The promoter regions of many genes that encode mitogenic and migratory factors expressed by SMCs in these lesions (Evanko et al, 1998; Murry et al, 1996; Ueda et al, 1996; Tanizawa et al, 1996; Rekhter & Gordon, 1994; Hughes et al, 1993; Brogi et al, 1993; Wilcox et al 1989; Wilcox et al, 1988) contain nucleotide (nt) recognition elements for the nuclear protein and transcription factor, Egr-1 (Khachigian & Collins, 1997; Khachigian et al, 1996). Egr-1 is not expressed in the unmanipulated artery wall, but is rapidly activated by mechanical injury (Khachigian et al, 1996; Silverman et al, 1997; Kim et al, 1995). It is also induced in vascular endothelial cells and/or SMCs exposed to fluid biomechanical forces (Khachigian et al, 1997; Sumpio et al, 1998) and multiple other pathophysiologically-relevant agonists (Delbridge & Khachigian, 1997).

SUMMARY OF THE INVENTION

Egr-1 (also known as NGFI-A and EGR-1) binds to the promoters of genes whose products influence cell movement and replication in the artery wall. Table 1 shows an alignment of the human EGR-1 cDNA sequence with the equivalent mouse (Egr-1) and rat (NGFI-A) sequences. The present inventors have now developed DNA-based enzymes that cut NGFI-A/Egr-1/EGR-1 RNA with high efficiency and specificity. The NGFI-A "DNAzyme" cleaved synthetic and *in vitro* transcribed NGFI-A RNA in a sequence-specific manner and inhibited production of NGFI-A in vascular smooth muscle cells without influencing levels of the related zinc finger protein, Sp1, or the immediate-early gene product, c-Fos. The DNAzyme blocked serum-inducible DNA synthesis in smooth muscle cells and attenuated total cell proliferation. The DNAzyme also inhibited the reparative response to mechanical injury, both in culture and in the rat carotid artery wall. These results indicate a necessary and sufficient role for NGFI-A/Egr-1/EGR-1 in vascular smooth muscle cell growth and provide the first demonstration of a DNAzyme targeted against NGFI-A/Egr-1/EGR-1 transcripts.

Accordingly, in a first aspect the present invention provides a DNAzyme which specifically cleaves EGR-1 mRNA, the DNAzyme including

(i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;

(ii) a first binding domain contiguous with the 5' end of the catalytic domain; and

(iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:1, such that the DNAzyme cleaves the EGR-1 mRNA.

As used herein, "DNAzyme" means a DNA molecule that specifically recognizes and cleaves a distinct target nucleic acid sequence, which may be either DNA or RNA.

In a preferred embodiment of the first aspect of the present invention, the binding domains are complementary to the regions immediately flanking the cleavage site. It will be appreciated by those skilled in the art, however, that strict complementarity may not be required for the DNAzyme to bind to and cleave the EGR-1 mRNA.

The catalytic domain of a DNAzyme of the present invention may be any suitable catalytic domain. Examples of suitable catalytic domains are described in *Santoro and Joyce, 1997* and US 5807718, the entire contents of which are incorporated herein by reference. In a preferred embodiment, the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA.

Within the parameters of the present invention, the binding domain lengths (also referred to herein as "arm lengths") can be of any permutation, and can be the same or different. In a preferred embodiment, the binding domain lengths are at least 6 nucleotides. Preferably, both binding domains have a combined total length of at least 14 nucleotides. Various permutations in the length of the two binding domains, such as 7+7, 8+8 and 9+9, are envisioned. It is well established that the greater the binding domain length, the more tightly it will bind to its complementary mRNA sequence. Accordingly, in a more preferred embodiment, each domain is nine or more nucleotides in length.

Within the context of the present invention, preferred cleavage sites within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 are as follows:

(i) the GU site corresponding to nucleotides 198-199;
(ii) the GU site corresponding to nucleotides 200-201;
(iii) the GU site corresponding to nucleotides 264-265;
(iv) the AU site corresponding to nucleotides 271-272;
(v) the AU site corresponding to nucleotides 292-293;
(vi) the AU site corresponding to nucleotides 301-302;
(vii) the GU site corresponding to nucleotides 303-304; and
(viii) the AU site corresponding to nucleotides 316-317.

In a further preferred embodiment, the DNAzyme has a sequence selected from:

(i) 5'-caggggacaGGCTAGCTACAACGAcgttgcggg
targets GU (bp 198, 199); arms hybridise to bp 189-207

(ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg
targets GU (bp 200, 201); arms hybridise to bp 191-209

(iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct
targets GU (bp 264, 265); arms hybridise to bp 255-273

(iv) 5'-ccgcggccaGGCTAGCTACAACGAcctggacga
targets AU (bp 271, 272); arms hybridise to bp 262-280

(v) 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt
targets AU (bp 271, 272); arms hybridise to bp 262-280

(vi) 5'-tcagctgcaGGCTAGCTACAACGActcggcctt
targets AU (bp 292-293); arms hybridise to bp 283-301

(vii) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat
targets AU (bp 301, 302); arms hybridise to bp 292-310

(viii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc
targets GU (bp 303, 304); arms hybridise to bp 294-312

(ix) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg
targets AU (bp 316, 317); arms hybridise to bp 307-325.

In a particularly preferred embodiment, the DNAzyme targets the AU site corresponding to nucleotides 271-272 (ie. the translation start codon).

In a further preferred embodiment, the DNAzyme has the sequence: 5'-ccgcggccaGGCTAGCTACAACGAcctggacga.

In applying DNAzyme-based treatments, it is preferable that the DNAzymes be as stable as possible against degradation in the intra-cellular milieu. One means of accomplishing this is by incorporating a 3'-3' inversion at one or more termini of the DNAzyme. More specifically, a 3'-3' inversion (also referred to herein simply as an "inversion") means the covalent phosphate bonding between the 3' carbons of the terminal nucleotide and its adjacent nucleotide. This type of bonding is opposed to the normal phosphate bonding between the 3' and 5' carbons of adjacent nucleotides, hence the term "inversion". Accordingly, in a preferred embodiment, the 3'-end nucleotide residue is inverted in the building domain contiguous with the 3' end of the catalytic domain. In addition to inversions, the instant DNAzymes may contain modified nucleotides. Modified nucleotides include, for example, N3'-P5' phosphoramidate linkages, and peptide-nucleic acid linkages. These are well known in the art.

In a particularly preferred embodiment, the DNAzyme includes an inverted T at the 3' position.

As will be appreciated by those skilled in the art, given that DNAzymes of the present invention cleave human EGR-1, similar DNAzymes can be produced to cleave the corresponding mRNA in other species, eg: rat (NGFI-A), mouse (Egr-1) etc. In a further aspect, the present invention provides such DNAzymes.

In a second aspect the present invention provides a pharmaceutical composition including a DNAzyme according to the first aspect and a pharmaceutically acceptable carrier.

In a third aspect the present invention provides a method of inhibiting EGR-1 activity in cells which includes exposing the cells to a DNAzyme according to the first aspect of the present invention.

In a fourth aspect the present invention provides a method of inhibiting proliferation or migration of cells in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

In a fifth aspect the present invention provides a method of treating a condition associated with cell proliferation or migration in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

In preferred embodiments of the third, fourth and fifth aspects of the present invention, the cells are vascular cells, particularly smooth muscle or endothelial cells. The cells may, however, be cells involved in neoplasia, such as tumour cells and the like.

Although the subject may be any animal or human, it is preferred that the subject is a human.

In a preferred embodiment, conditions associated with SMC proliferation (and migration) are selected from post-angioplasty restenosis, vein graft failure, transplant coronary disease and complications associated with atherosclerosis (cerebrovascular infarction (stroke), myocardial infarction (heart attack) or peripheral vascular disease (gangrene of the extremities).

Within the parameters of the fourth and fifth aspects of the present invention, any suitable mode of administration may be used to administer or deliver the DNAzyme.

In particular, delivery of the nucleic acid agents described may be achieved by one or more of the following methods:

(a) Liposomes and liposome-protein conjugates and mixtures.

(b) Using catheters to deliver intra-luminal formulations of the nucleic acid as a solution or in a complex with a liposome.

(c) Catheter delivery to adventitial tissue as a solution or in a complex with a liposome.

(d) Within a polymer such as Pluronic gels or within ethylene vinyl acetate copolymer (EVAc). The polymer will be delivered intra-luminally.

(e) Within a viral-liposome complex, such as Sendai virus.

(f) The nucleic acid may be delivered by a double angioplasty balloon device fixed to catheter.

(g) The nucleic acid could be delivered on a specially prepared stent of the Schatz-Palmaz or derivative type. The stent could be coated with a polymer or agent impregnated with nucleic acid that allows controlled release of the molecules at the vessel wall.

In a preferred embodiment, the mode of administration is topical administration. Topical administration may be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The topical administration can be performed, for example, via catheter and topical injection, and via coated stent as discussed below.

Pharmaceutical carriers for topical administration are well known in the art, as are methods for combining same with active agents to be delivered. The following delivery systems, which employ a number of routinely used carriers, are only representative of the many embodiments envisioned for administering the instant composition.

Topical delivery systems include, for example, gels and solutions, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In the preferred embodiment, the pharmaceutically acceptable carrier is a liposome or a biodegradable polymer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N^{I},N^{II},N^{III}-tetramethyl-N,N^{I},N^{II},N^{III}-tetrapalmitylspermine and dioleoyl phosphatidyl-ethanolamine (DOPE) (GIBCO BRL); (2) Cytofection GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-trimethyl-ammoniummethylsulfate) (Boehringer Mannheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

Determining the prophylactically effective dose of the instant pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the prophylactically effective does contains between about 0.1 mg and about 1 g of the instant DNAzyme. In another embodiment, the prophylactically effective dose contains between

about 1 mg and about 100 mg of the instant DNAzyme. In a further embodiment, the prophylactically effective does contains between about 10 mg and about 50 mg of the instant DNAzyme. In yet a further embodiment, the prophylactically effective does contains about 25 mg of the instant DNAzyme.

In a sixth aspect the present invention provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an angioplastic stent operably coated with a prophylactically effective dose of a DNAzyme according to the first aspect.

Angioplastic stents, also known by other terms such as "intravascular stents" or simple "stents", are well known in the art. They are routinely used to prevent vascular closure due to physical anomalies such as unwanted inward growth of vascular tissue due to surgical trauma. They often have a tubular, expanding lattice-type structure appropriate for their function, and can optionally be biodegradable.

In this invention, the stent can be operably coated with the instant pharmaceutical composition using any suitable means known in the art. Here, "operably coating" a stent means coating it in a way that permits the timely release of the pharmaceutical composition into the surrounding tissue to be treated once the coated stent is administered. Such coating methods, for example, can use the polymer polypyrrole.

In a seventh aspect, the present invention provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a stent according to the fifth aspect to the subject at around the time of the angioplasty.

As used herein, administration "at around the time of angioplasty" can be performed during the procedure, or immediately before or after the procedure. The administering can be performed according to known methods such as catheter delivery.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting Figures and Examples.

Table 1

```
Symbol comparison table: GenRunData:pileupdna.cmp  CompCheck: 6876
                 GapWeight: 5.000
           GapLengthWeight: 0.300
EGR1align.msf  MSF: 4388  Type: N  April  7, 1998 12:07  Check: 5107
Name: mouseEGR1          Len:  4388  Check: 8340  Weight:  1.00
Name: ratEGR1            Len:  4388  Check: 8587  Weight:  1.00
Name: humanEGR1          Len:  4388  Check: 8180  Weight:  1.00

NB. THIS IS RAT NGFI-A numbering
                                                                      50
                    1
mouseEgr1  .......... .......... .......... .......... ..........
  ratNGFIA CCGCGGAGCC TCAGCTCTAC GCGCCTGGCG CCCTCCCTAC GCGGGCGTCC
humanEGR1  .......... .......... .......... .......... ..........
                                                                     100
                   51
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  CCGACTCCCG CGCGCGTTCA GGCTCCGGGT TGGGAACCAA GGAGGGGGAG
humanEGR1  .......... .......... .......... .......... ..........
                                                                     150
                  101
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  GGTGGGTGCG CCGACCCGGA AACACCATAT AAGGAGCAGG AAGGATCCCC
humanEGR1  .......... .......... .......... .......... ..........
                                                                     200
                  151
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  CGCCGGAACA GACCTTATTT GGGCAGCGCC TTATATGGAG TGGCCCAATA
humanEGR1  .......... .......... .......... .......... ..........
                                                                     250
                  201
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  TGGCCCTGCC GCTTCCGGCT CTGGGAGGAG GGGCGAACGG GGGTTGGGGC
humanEGR1  .......... .......... .......... .......... ..........
                                                                     300
                  251
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  GGGGGCAAGC TGGGAACTCC AGGAGCCTAG CCCGGGAGGC CACTGCCGCT
humanEGR1  .......... .......... .......... .......... ..........
                                                                     350
                  301
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  GTTCCAATAC TAGGCTTTCC AGGAGCCTGA GCGCTCAGGG TGCCGGAGCC
humanEGR1  .......... .......... .......... .......... ..........
                                                                     400
                  351
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  GGTCGCAGGG TGGAAGCGCC CACCGCTCTT GGATGGGAGG TCTTCACGTC
humanEGR1  .......... .......... .......... .......... ..........
                                                                     450
                  401
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  ACTCCGGGTC CTCCCGGTCG GTCCTTCCAT ATTAGGGCTT CCTGCTTCCC
humanEGR1  .......... .......... .......... .......... ..........
                                                                     500
                  451
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  ATATATGGCC ATGTACGTCA CGGCGGAGGC GGGCCCGTGC TGTTTCAGAC
```

```
humanEGR1  .......... .......... .......... .......... ..........
                                                              550
           501
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  CCTTGAAATA GAGGCCGATT CGGGGAGTCG CGAGAGATCC CAGCGCGCAG
humanEGR1  .......... .......... .......... .......... ....CCGCAG
                                                              600
           551
mouseEGR1  .....GGGGA GCCGCCGCCG CGATTCGCCG CCGCCGCCAG CTTCCGCCGC
  ratEGR1  AACTTGGGGA GCCGCCGCCG CGATTCGCCG CCGCCGCCAG CTTCCGCCGC
humanEGR1  AACTTGGGGA GCCGCCGCCG CCATCCGCCG CCGCAGCCAG CTTCCGCCGC
                                                              650
           601
mouseEGR1  CGCAAGATCG GCCCCTGCCC CAGCCTCCGC GGCAGCCCTG CGTCCACCAC
  ratEGR1  CGCAAGATCG GCCCCTGCCC CAGCCTCCGC GGCAGCCCTG CGTCCACCAC
humanEGR1  CGCAGGACCG GCCCCTGCCC CAGCCTCCGC AGCCGCGGCG CGTCCACGCC
                                                              700
           651
mouseEGR1  GGGCCGCGGC TACCGCCAGC CTGGGGGCCC ACCTACACTC CCCGCAGTGT
  ratEGR1  GGGCCGCGGC CACCGCCAGC CTGGGGGCCC ACCTACACTC CCCGCAGTGT
humanEGR1  CGCCCGCGCC CAGGGCGAGT CGGGGTCGCC GCCTGCACGC TTCTCAGTGT
                                                              750
           701
mouseEGR1  GCCCCTGCAC CCCGCATGTA ACCCGGCCAA CCCCCGGCGA GTGTGCCCTC
  ratEGR1  GCCCCTGCAC CCCGCATGTA ACCCGGCCAA CATCCGGCGA GTGTGCCCTC
humanEGR1  TCCCC.GCGC CCCGCATGTA ACCCGGCCAG GCCCCCGCAA CGGTGTCCCC
                                                              800
           751
mouseEGR1  AGTAGCTTCG GCCCCGGGCT GCGCCCACC. .ACCCAACAT CAGTTCTCCA
  ratEGR1  AGTAGCTTCG GCCCCGGGCT GCGCCCACC. .ACCCAACAT CAGCTCTCCA
humanEGR1  TGCAGCTCCA GCCCCGGGCT GCACCCCCCC GCCCCGACAC CAGCTCTCCA
                                                              850
           801
mouseEGR1  GCTCGCTGGT CCGGGATGGC AGCGGCCAAG GCCGAGATGC AATTGATGTC
  ratEGR1  GCTCGCACGT CCGGGATGGC AGCGGCCAAG GCCGAGATGC AATTGATGTC
humanEGR1  GCCTGCTCGT CCAGGATGGC CGCGGCCAAG GCCGAGATGC AGCTGATGTC

           ED5 (rat) arms hybridise to bp 807-825 in rat sequ
           hED5(hum) arms hybridise to bp 262-280 in hum sequ

                                                              900
           851
mouseEGR1  TCCGCTGCAG ATCTCTGACC CGTTCGGCTC CTTTCCTCAC TCACCCACCA
  ratEGR1  TCCGCTGCAG ATCTCTGACC CGTTCGGCTC CTTTCCTCAC TCACCCACCA
humanEGR1  CCCGCTGCAG ATCTCTGACC CGTTCGGATC CTTTCCTCAC TCGCCCACCA
                                                              950
           901
mouseEGR1  TGGACAACTA CCCCAAACTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT
  ratEGR1  TGGACAACTA CCCCAAACTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT
humanEGR1  TGGACAACTA CCCTAAGCTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT
                                                             1000
           951
mouseEGR1  CCCCAGTTCC TCGGTGCTGC CGGAACCCCA GAGGGCAGCG GCGGTAAT..
  ratEGR1  CCCCAGTTCC TCGGTGCTGC CGGAACCCCA GAGGGCAGCG GCGGCAATAA
humanEGR1  CCCCAGTTCC TCGGCGCCGC CGGGGCCCCA GAGGGCAGCG GCAGCAACAG
                                                             1050
           1001
mouseEGR1  .......AGC AGCAGCAGCA CCAGCAGCGG GGGCGGTGGT GGGGGCGGCA
  ratEGR1  CAGCAGCAGC AGCAGCAGCA GCAGCAGCGG GGGCGGTGGT GGGGGCGGCA
humanEGR1  CAGCAGCAGC AGCAGCGGGG GCGGTGGAGG CGGCGGGGGC GGCAGCAACA
```

```
                                                                    1100
                1051
mouseEGR1   GCAACAGCGG CAGCAGCGCC TTCAATCCTC AAGGGGAGCC GAGCGAACAA
  ratEGR1   GCAACAGCGG CAGCAGCGCT TTCAATCCTC AAGGGGAGCC GAGCGAACAA
humanEGR1   GCAGCAGCAG CAGCAGCACC TTCAACCCTC AGGCGGACAC GGGCGAGCAG
                                                                    1150
                1101
mouseEGR1   CCCTATGAGC ACCTGACCAC AG...AGTCC TTTTCTGACA TCGCTCTGAA
  ratEGR1   CCCTACGAGC ACCTGACCAC AGGTAAGCGG TGGTCTGCGC CGAGGCTGAA
humanEGR1   CCCTACGAGC ACCTGACCGC AG...AGTCT TTTCCTGACA TCTCTCTGAA
                                                                    1200
                1151
mouseEGR1   TAATGAGAAG GCGATGGTGG AGACGAGTTA TCCCAGCCAA ACGACTCGGT
  ratEGR1   TCCCCCTTCG TGACTACCCT AACGTCCAGT CCTTTGCAGC ACGGACCTGC
humanEGR1   CAACGAGAAG GTGCTGGTGG AGACCAGTTA CCCCAGCCAA ACCACTCGAC
                                                                    1250
                1201
mouseEGR1   TGCCTCCCAT CACCTATACT GGCCGCTTCT CCCTGGAGCC CGCACCCAAC
  ratEGR1   ATCTAGATCT TAGGGACGGG ATTGGGATTT CCCTCTATTC ..CACACAGC
humanEGR1   TGCCCCCCAT CACCTATACT GGCCGCTTTT CCCTGGAGCC TGCACCCAAC
                                                                    1300
                1251
mouseEGR1   AGTGGCAACA CTTTGTGGCC TGAACCCCTT TTCAGCCTAG TCAGTGGCCT
  ratEGR1   TCCAGGGACT TGTGTTAGAG GGATGTCTGG GGACCCCCCA ACCCTCCATC
humanEGR1   AGTGGCAACA CCTTGTGGCC CGAGCCCCTC TTCAGCTTGG TCAGTGGCCT
                                                                    1350
                1301
mouseEGR1   CGTGAGCATG ACCAATCCTC CGACCTCTTC ATCCTCGGCG CCTTCTCCAG
  ratEGR1   CTTGCGGGTG CGCGGAGGGC AGACCGTTTG TTTTGGATGG AGAACTCAAG
humanEGR1   AGTGAGCATG ACCAACCCAC CGGCCTCCTC GTCCTCAGCA CCATCTCCAG
                                                                    1400
                1351
mouseEGR1   CTGCTTCATC GTCTTCCTCT GCCTCCCAGA GCCCGCCCCT GAGCTGTGCC
  ratEGR1   TTGCGTGGGT GGCT...... .....GGAGT GGGGGAGGGT TTGTTTTGAT
humanEGR1   CGGCCTCCTC CGC...CTCC GCCTCCCAGA GCCCACCCCT GAGCTGCGCA
                                                                    1450
                1401
mouseEGR1   GTGCCGTCCA ACGACAGCAG TCCCATCTAC TCGGCTGCGC CCACCTTTCC
  ratEGR1   GAGCAGGGTT GC....CCCC TCCCCCGCGC GCGTTGTCGC GAGCCTTGTT
humanEGR1   GTGCCATCCA ACGACAGCAG TCCCATTTAC TCAGCGGCAC CCACCTTCCC
                                                                    1500
                1451
mouseEGR1   TACTCCCAAC ACTGACATTT TTCCTGAGCC CCAAAGCCAG GCCTTTCCTG
  ratEGR1   TGCAGCTTGT TCCCAAGGAA GGGCTGAAAT CTGTCACCAG GGATGTCCCG
humanEGR1   CACGCCGAAC ACTGACATTT TCCCTGAGCC ACAAAGCCAG GCCTTCCCGG
                                                                    1550
                1501
mouseEGR1   GCTCGGCAGG CACAGCCTTG CAGTACCCGC CTCCTGCCTA CCCTGCCACC
  ratEGR1   CCGCCCAGGG TAGGGGCGCG CATTAGCTGT GGCC.ACTAG GGTGCTGGCG
humanEGR1   GCTCGGCAGG GACAGCGCTC CAGTACCCGC CTCCTGCCTA CCCTGCCGCC
                                                                    1600
                1551
mouseEGR1   AAAGGTGGTT TCCAGGTTCC CATGATCCCT GACTATCTGT TTCCACAACA
  ratEGR1   GGATTCCCTC ACCCCGGACG CCTGCTGCGG AGCGCTCTCA GAGCTGCAGT
humanEGR1   AAGGGTGGCT TCCAGGTTCC CATGATCCCC GACTACCTGT TTCCACAGCA
                                                                    1650
                1601
mouseEGR1   ACAGGGAGAC CTGAGCCTGG GCACCCCAGA CCAGAAGCCC TTCCAGGGTC
  ratEGR1   AGAGGGGGAT TCTCTGTTTG CGTCAGCTGT CGAAATGGCT CT......GC
humanEGR1   GCAGGGGGAT CTGGGCCTGG GCACCCCAGA CCAGAAGCCC TTCCAGGGCC
```

```
                                                                    1700
            1651
mouseEGR1   TGGAGAACCG TACCCAGCAG CCTTCGCTCA CTCCACTATC CACTATTAAA
  ratEGR1   CACTGGAGCA GGTCCAGGAA CATTGCAATC TGCTGCTATC AATTATTAAC
humanEGR1   TGGAGAGCCG CACCCAGCAG CCTTCGCTAA CCCCTCTGTC TACTATTAAG

                                                                    1750
            1701
mouseEGR1   GCCTTCGCCA CTCAGTCGGG CTCCCAGGAC TTAAAG.... ...GCTCTTA
  ratEGR1   CACATCGAGA GTCAGTGGTA GCCGGGCGAC CTCTTGCCTG GCCGCTTCGG
humanEGR1   GCCTTTGCCA CTCAGTCGGG CTCCCAGGAC CTGAAG.... ...GCCCTCA

                                                                    1800
            1751
mouseEGR1   ATACCACCTA CCAATCCCAG CTCATCA..A ACCCAGCCGC ATGCGCAAGT
  ratEGR1   CTCTCATCGT CCAGTGATTG CTCTCCAGTA ACCAGGCCTC TCTGTTCTCT
humanEGR1   ATACCAGCTA CCAGTCCCAG CTCATCA..A ACCCAGCCGC ATGCGCAAGT

                                                                    1850
            1801
mouseEGR1   ACCCCAACCG GCCCAGCAAG ACACCCCCCC ATGAACGCCC ATATGCTTGC
  ratEGR1   TTCCTGCCAG AGTCCTTTTC TGACATCGCT CTGAATAACG AGAAG..GCG
humanEGR1   ATCCCAACCG GCCCAGCAAG ACGCCCCCCC ACGAACGCCC TTACGCTTGC

                                                                    1900
            1851
mouseEGR1   CCTGTCGAGT CCTGCGATCG CCGCTTTTCT CGCTCGGATG AGCTTACCCG
  ratEGR1   CTGGTGGAGA CAAGTTATCC CAGCCAAACT ACCCGGTTGC CTCCCATCAC
humanEGR1   CCAGTGGAGT CCTGTGATCG CCGCTTCTCC CGCTCCGACG AGCTCACCCG

                                                                    1950
            1901
mouseEGR1   CCATATCCGC ATCCACACAG GCCAGAAGCC CTTCCAGTGT CGAATCTGCA
  ratEGR1   CTATACTGGC CGCTTCTCCC TGGAGCCTGC ACCCAACAGT GGCAACACTT
humanEGR1   CCACATCCGC ATCCACACAG GCCAGAAGCC CTTCCAGTGC CGCATCTGCA

                                                                    2000
            1951
mouseEGR1   TGCGTAACTT CAGTCGTAGT GACCACCTTA CCACCCACAT CCGCACCCAC
  ratEGR1   TGTGGCCTGA ACCCCTTTTC AGCCTAGTCA GTGGCCTTGT GAGCATGACC
humanEGR1   TGCGCAACTT CAGCCGCAGC GACCACCTCA CCACCCACAT CCGCACCCAC

                                                                    2050
            2001
mouseEGR1   ACAGGCGAGA AGCCTTTTGC CTGTGACATT TGTGGGAGGA AGTTTGCCAG
  ratEGR1   AACCCTCCAA CCTCTTCATC CTCAGCGCCT TCTCCAGCTG CTTCATCGTC
humanEGR1   ACAGGCGAAA AGCCCTTCGC CTGCGACATC TGTGGAAGAA AGTTTGCCAG

                                                                    2100
            2051
mouseEGR1   GAGTGATGAA CGCAAGAGGC ATACCAAAAT CCATTTAAGA CAGAAGGACA
  ratEGR1   TTCCTCTGCC TCCCAGAGCC CACCCCTGAG CTGTGCCGTG CCGTCCAACG
humanEGR1   GAGCGATGAA CGCAAGAGGC ATACCAAGAT CCACTTGCGG CAGAAGGACA

                                                                    2150
            2101
mouseEGR1   AGAAAGCAGA CAAAAGTGTG GTGGCCTCCC CGGCTGC... .CTCTTCACT
  ratEGR1   ACAGCAGTCC CATTTACTCA GCTGCACCCA CCTTTCCTAC TCCCAACACT
humanEGR1   AGAAAGCAGA CAAAAGTGTT GTGGCCTCTT CGGCCACCTC CTCTCTCTCT

                                                                    2200
            2151
mouseEGR1   .......... .......... CTCTTCTTAC CCATCCCCAG TGGCTACCTC
  ratEGR1   .......... .......... GACATTTTTC CTGAGCCCCA AAGCCAGGCC
humanEGR1   TCCTACCCGT CCCCGGTTGC TACCTCTTAC CCGTCCCCGG TTACTACCTC

                                                                    2250
            2201
mouseEGR1   CTACCCATCC CCTGCCACCA CCTCATTCCC ATCCCCTGTG CCCACTTCCT
  ratEGR1   TTTCCTGGCT CTGCAGGCAC AGCCTTGCAG TACCCGCCTC CTGCCTACCC
humanEGR1   TTATCCATCC CCGGCCACCA CCTCATACCC ATCCCCTGTG CCCACCTCCT
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                                                                    2300
                2251
mouseEGR1       ACTCCTCTCC TGGCTCCTCC ACCTACCCAT CTCCTGCGCA CAGTGGCTTC
  ratEGR1       TGCCACCAAG GGTGGTTTCC AGGTTCCCAT GATCCCTGAC TATCTGTTTC
humanEGR1       TCTCCTCTCC CGGCTCCTCG ACCTACCCAT CCCCTGTGCA CAGTGGCTTC
                                                                    2350
                2301
mouseEGR1       CCGTCGCCGT CAGTGGCCAC CACCTTTGCC TCCGTTCC.. ..........
  ratEGR1       CACAACAACA GGGAGACCTG AGCCTGGGCA CCCCAGACCA GAAGCCCTTC
humanEGR1       CCCTCCCCGT CGGTGGCCAC CACGTACTCC TCTGTTCCC. ..........
                                                                    2400
                2351
mouseEGR1       ....ACCTGC TTTCCCCACC CAGGTCAGCA GCTTCCCGTC TGCGGGCGTC
  ratEGR1       CAGGGTCTGG AGAACCGTAC CCAGCAGCCT TCGCTCACTC CACTATCCAC
humanEGR1       .....CCTGC TTTCCCGGCC CAGGTCAGCA GCTTCCCTTC CTCAGCTGTC
                                                                    2450
                2401
mouseEGR1       AGCAGCTCCT TCAGCACCTC AACTGGTCTT TCAGACATGA CAGCGACCTT
  ratEGR1       TATCAAAGCC TTCGCCACTC AGTCGGGCTC CCAGGACTTA AAGGCTCTTA
humanEGR1       ACCAACTCCT TCAGCGCCTC CACAGGGCTT TCGGACATGA CAGCAACCTT
                                                                    2500
                2451
mouseEGR1       TTCTCCCAGG ACAATTGAAA TTTGCTAAAG GGA....... .ATAAAAG..
  ratEGR1       ATAACACCTA CCAGTCCCAA CTCATCAAAC CCAGCCGCAT GCGCAAGT..
humanEGR1       TTCTCCCAGG ACAATTGAAA TTTGCTAAAG GGAAAGGGGA AAGAAAGGGA
                                                                    2550
                2501
mouseEGR1       .AAAGCAAAG GGAGAGGCAG GAAAGACATA AAAGCA...C AGGAGGGAAG
  ratEGR1       .ACCCCAACC GGCCCAGCAA GACACCCCCC CATGAACGCC CGTATGCTTG
humanEGR1       AAAGGGAGAA AAAGAAACAC AAGAGACTTA AAGGACAGGA GGAGGAGATG
                                                                    2600
                2551
mouseEGR1       AGATGGCCGC AAGAGGGGCC ACCTCTTAGG TCAGATGGAA GATCTCAGAG
  ratEGR1       CCCTGTTGAG TCCTGCGATC GCCGCTTTTC TCGCTCGGAT GAGCTTACAC
humanEGR1       GCCATAGGAG AGGAGGGTT. .CCTCTTAGG TCAGATGGAG GTTCTCAGAG
                                                                    2650
                2601
mouseEGR1       CCAAGTCCTT CTACTCACGA GTA..GAAGG ACCGTTGGCC AACAGCCCTT
  ratEGR1       GCCACATCCG CATCCATACA GGC..CAGAA GCCCTTCCAG TGTCGAATCT
humanEGR1       CCAAGTCCTC CCTCTCTACT GGAGTGGAAG GTCTATTGGC CAACAATCCT
                                                                    2700
                2651
mouseEGR1       TCACTTACCA TCCCTGCCTC CCCCGTCCTG TTCCCTTTGA CTTCAGCTGC
  ratEGR1       GCATGCGTAA TTTCAGTCGT AGTGACCACC TTACCACCCA CATCCGCACC
humanEGR1       TTCTGCCCAC TTCCCCTTCC CCAATTACTA TTCCCTTTGA CTTCAGCTGC
                                                                    2750
                2701
mouseEGR1       CTGAAACAGC CATGTCCAAG TTCTTCACCT CTATCCAAAG GACTTGATTT
  ratEGR1       C..ACACAGG CGAGAAGCCT TTTGCCTGTG ACATTTGTGG GAGAAAGTTT
humanEGR1       CTGAAACAGC CATGTCCAAG TTCTTCACCT CTATCCAAAG AACTTGATTT
                                                                    2800
                2751
mouseEGR1       GCATGG.... ..TATTGGAT AAATCATTTC AGTATCCTCT ..........
  ratEGR1       GCCAGGAGTG ATGAACGCAA GAGGCATACC AAAATCCACT TAAGACAGAA
humanEGR1       GCATGGA... ..TTTTGGAT AAATCATTTC AGTATCATCT ..........
                                                                    2850
                2801
mouseEGR1       .....CCATC ACATGCCTGG CCCTTGCTCC CTTCAGCGCT AGACCATCAA
  ratEGR1       GGACAAGAAA GCAGACAAAA GTGTCGTGGC CTCCTCAGCT GCCTCTTCCC
humanEGR1       ....CCATCA TATGCCTGAC CCCTTGCTCC CTTCAATGCT AGAAAATCGA
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                                                                    2900
            2851
mouseEGR1   GTTGGCATAA AGAAAAAAAA ATGGGTTTGG GCCCTCAGAA CCCTGCCCTG
  ratEGR1   TCTCTTCCTA CCCATCCCCA GTGGCTACCT CCTACCCATC CCCCGCCACC
humanEGR1   GTTGGC.... .....AAAAT GGGGTTTGGG CCCCTCAGAG CCCTGCCCTG
                                                                    2950
            2901
mouseEGR1   CATCTTTGTA CAGCATCTGT GCCATGGATT TTGTTTTCCT TGGGGTATTC
  ratEGR1   ACCTCATTTC CATCCCCAGT GCCCACCTCT TACTCCTCTC CGGGCTCCTC
humanEGR1   CACCCTTGTA CAGTGTCTGT GCCATGGATT TCGTTTTTCT TGGGGTACTC
                                                                    3000
            2951
mouseEGR1   TTGATGTGAA GATAATTTGC ATACT..... .CTATTGTAT TATTTGGAGT
  ratEGR1   TACCTACCCG TCTCCTGCAC ACAGTGGCTT CCCATCGCCC TCGGTGGCCA
humanEGR1   TTGATGTGAA GATAATTTGC ATATT..... .CTATTGTAT TATTTGGAGT
                                                                    3050
            3001
mouseEGR1   TAAATCCTCA CTTTGGGG.. GAGGGGGGAG CAAAGCCAAG CAAACCAATG
  ratEGR1   CCACCTATGC CTCCGTCC.. CACCTGCTTT CCCTGCCCAG GTCAGCACCT
humanEGR1   TAGGTCCTCA CTTGGGGGAA AAAAAAAAAA AAAAGCCAAG CAAACCAATG
                                                                    3100
            3051
mouseEGR1   ATGATCCTCT ATTTTGTGAT GACTCTGCTG TGACATTA.. ..........
  ratEGR1   TCCAGTCTGC AGGGGTCAGC AACTCCTTCA GCACCTCAAC GGGTCTTTCA
humanEGR1   GTGATCCTCT ATTTTGTGAT GATGCTGTGA CAATA..... ..........
                                                                    3150
            3101
mouseEGR1   .GGTTTGAAG CATTTTTTTT TTCAAGCAGC AGTCCTAGGT ATTAACTGGA
  ratEGR1   GACATGACAG CAACCTTTTC TCCTAGGACA ATTGAAATTT GCTAAAGGGA
humanEGR1   ...AGTTTGA ACCTTTTTTT TTGAAACAGC AGTCCCAG.. ..TATTCTCA
                                                                    3200
            3151
mouseEGR1   ..GCATGTGT CAGAGTGTTG TTCCGTTAAT TTTGTAAATA CTGGCTCGAC
  ratEGR1   ATGAAAGAGA GCAAAGGGAG GGGAGCGCGA GAGACAATAA AGGACAGGAG
humanEGR1   GAGCATGTGT CAGAGTGTTG TTCCGTTAAC CTTTTTGTAA ATACTGCTTG
                                                                    3250
            3201
mouseEGR1   .TGTAACTCT CACATGTGAC AAAGTATGGT TTGTTTGGTT GGGTTTTGTT
  ratEGR1   .GGAAGAAAT GGCCCGCAAG AGGGGCTGCC TCTTAGGTCA GATGGAAGAT
humanEGR1   ACCGTACTCT CACATGTGGC AAAATATGGT TTGGTTTTTC TTTTTTTTTT
                                                                    3300
            3251
mouseEGR1   TTTGAGAATT TTTTTGCCCG TCCCTTTGGT TTCAAAAGTT TCACGTCTTG
  ratEGR1   CTCAGAGCCA AGTCCTTCTA GTCAGTAGAA GGCCCGTTGG CCACCAGCCC
humanEGR1   TTGAAAGTGT TTTTTCTTCG TCCTTTTGGT TTAAAAAGTT TCACGTCTTG
                                                                    3350
            3301
mouseEGR1   GTGCCTTTTG TGTGACACGC CTT.CCGATG GCTTGACATG CGCA......
  ratEGR1   TTTCACTTAG CGTCCCTGCC CTC.CCCAGT CCCGGTCCTT TTGACTTCAG
humanEGR1   GTGCCTTTTG TGTGATGCCC CTTGCTGATG GCTTGACATG TGCAAT....
                                                                    3400
            3351
mouseEGR1   ...GATGTGA GGGACACGCT CACCTTAGCC TTAA...GGG GGTAGGAGTG
  ratEGR1   CTGCCTGAAA CAGCCACGTC CAAGTTCTTC ACCT...CTA TCCAAAGGAC
humanEGR1   .....TGTGA GGGACATGCT CACCTCTAGC CTTAAGGGGG GCAGGGAGTG
                                                                    3450
            3401
mouseEGR1   ATGTGTTGGG GGAGGCTTGA GAGCAAAAAC GAGGAAGAGG GCTGAGCTGA
  ratEGR1   TTGATTTGCA TGGTATTGGA TAAACCATTT CAGCATCATC TCCACCACAT
humanEGR1   ATGATTTGGG GGAGGCTTTG GGAGCAAAAT AAGGAAGAGG GCTGAGCTGA
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                                                                    3500
              3451
mouseEGR1  GCTTTCGGTC TCCAGAATGT AAGAAGAAAA AATTTAAACA AAAATCTGAA
  ratEGR1  GCCTGGCCCT TGCTCCCTTC AGCACTAGAA CATCAAGTTG GCTGAAAAAA
humanEGR1  GCTTCGGTTC TCCAGAATGT AAGAAAACAA AATCTAAAAC AAAATCTGAA
                                                                    3550
              3501
mouseEGR1  CTCTCAAAAG TCTATTTTTC TAAACTGAAA ATGTAAATTT ATACATCTAT
  ratEGR1  AAAATGGGTC TGGGCCCTCA GAACCCTGCC CTGTATCTTT GTACA.....
humanEGR1  CTCTCAAAAG TCTATTTTTT TAA.CTGAAA ATGTAAATTT ATAAATATAT
                                                                    3600
              3551
mouseEGR1  TCAGGAGTTG GAGTGTTGTG GTTACCTACT GAGTAGGCTG CAGTTTTTGT
  ratEGR1  GCATCTGTGC CATGGATTTT GTTTTCCTTG GGGTATTCTT GATGTGAAGA
humanEGR1  TCAGGAGTTG GAATGTTGTA GTTACCTACT GAGTAGGCGG CGATTTTTGT
                                                                    3650
              3601
mouseEGR1  ATGTTATGAA CATGAAGTTC ATTATTTTGT GGTTTTATTT TACTTTGTAC
  ratEGR1  TAATTTGCAT ACTCTATTGT ACTATTTGGA GTTAAATTCT CACTTTGGGG
humanEGR1  ATGTTATGAA CATGCAGTTC ATTATTTTGT GGTTCTATTT TACTTTGTAC
                                                                    3700
              3651
mouseEGR1  TTGTGTTTGC TTAAACAAAG TAACCTGTTT GGCTTATAAA CACATTGAAT
  ratEGR1  GAGGGGGAGC AAAGCCAAGC AAACCAATGG TGATCCTCTA TTTTGTGATG
humanEGR1  TTGTGTTTGC TTAAACAAAG TGA.CTGTTT GGCTTATAAA CACATTGAAT
                                                                    3750
              3701
mouseEGR1  GCGCTCTATT GCCCATGG.. ..GATATGTG GTGTGTATCC TTCAGAAAAA
  ratEGR1  ATCCTGCTGT GACATTAGGT TTGAAACTTT TTTTTTTTTT TGAAGCAGCA
humanEGR1  GCGCTTTATT GCCCATGG.. ..GATATGTG GTGTATATCC TTCCAAAAAA
                                                                    3800
              3751
mouseEGR1  TTAAAAGGAA AAAT..... ..........  .......... ..........
  ratEGR1  GTCCTAGGTA TTAACTGGAG CATGTGTCAG AGTGTTGTTC CGTTAATTTT
humanEGR1  TTAAAACGAA AATAAAGTAG CTGCGATTGG G.........  ..........
                                                                    3850
              3801
mouseEGR1  ..........  ..........  ..........  ..........  ..........
  ratEGR1  GTAAATACTG CTCGACTGTA ACTCTCACAT GTGACAAAAT ACGGTTTGTT
humanEGR1  ..........  ..........  ..........  ..........  ..........
                                                                    3900
              3851
mouseEGR1  ..........  ..........  ..........  ..........  ..........
  ratEGR1  TGGTTGGGTT TTTTGTTGTT TTTGAAAAAA AAATTTTTTT TTTGCCCGTC
humanEGR1  ..........  ..........  ..........  ..........  ..........
                                                                    3950
              3901
mouseEGR1  ..........  ..........  ..........  ..........  ..........
  ratEGR1  CCTTTGGTTT CAAAAGTTTC ACGTCTTGGT GCCTTTGTGT GACACACCTT
humanEGR1  ..........  ..........  ..........  ..........  ..........
                                                                    4000
              3951
mouseEGR1  ..........  ..........  ..........  ..........  ..........
  ratEGR1  GCCGATGGCT GGACATGTGC AATCGTGAGG GGACACGCTC ACCTCTAGCC
humanEGR1  ..........  ..........  ..........  ..........  ..........
                                                                    4050
              4001
mouseEGR1  ..........  ..........  ..........  ..........  ..........
  ratEGR1  TTAAGGGGGT AGGAGTGATG TTTCAGGGGA GGCTTTAGAG CACGATGAGG
humanEGR1  ..........  ..........  ..........  ..........  ..........
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                                                                    4100
              4051
mouseEGR1     .......... .......... .......... .......... ..........
  ratEGR1     AAGAGGGCTG AGCTGAGCTT TGGTTCTCCA GAATGTAAGA AGAAAAATTT
humanEGR1     .......... .......... .......... .......... ..........
                                                                    4150
              4101
mouseEGR1     .......... .......... .......... .......... ..........
  ratEGR1     AAAACAAAAA TCTGAACTCT CAAAAGTCTA TTTTTTTAAC TGAAAATGTA
humanEGR1     .......... .......... .......... .......... ..........
                                                                    4200
              4151
mouseEGR1     .......... .......... .......... .......... ..........
  ratEGR1     GATTTATCCA TGTTCGGGAG TTGGAATGCT GCGGTTACCT ACTGAGTAGG
humanEGR1     .......... .......... .......... .......... ..........
                                                                    4250
              4201
mouseEGR1     .......... .......... .......... .......... ..........
  ratEGR1     CGGTGACTTT TGTATGCTAT GAACATGAAG TTCATTATTT TGTGGTTTTA
humanEGR1     .......... .......... .......... .......... ..........
                                                                    4300
              4251
mouseEGR1     .......... .......... .......... .......... ..........
  ratEGR1     TTTTACTTCG TACTTGTGTT TGCTTAAACA AAGTGACTTG TTTGGCTTAT
humanEGR1     .......... .......... .......... .......... ..........
                                                                    4350
              4301
mouseEGR1     .......... .......... .......... .......... ..........
  ratEGR1     AAACACATTG AATGCGCTTT ACTGCCCATG GGATATGTGG TGTGTATCCT
humanEGR1     .......... .......... .......... .......... ..........
                                                          4388
              4351
mouseEGR1     .......... .......... .......... ........
  ratEGR1     TCAGAAAAAT TAAAAGGAAA ATAAAGAAAC TAACTGGT
humanEGR1     .......... .......... .......... ........
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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 *In vitro* cleavage of NGFI-A RNA. **a**, Sequence of NGFI-A DNAzyme (ED5), its scrambled control (ED5SCR) and 23 nt synthetic rat substrate. The translational start site is underlined. **b**, Time-dependent and sequence-specific cleavage of synthetic substrate by NGFI-A DNAzyme. The 12 nt reaction product is shown. **c**, Dose-dependent cleavage by ED5. The DNAzyme to substrate stoichiometric ratio is indicated. Sequence of hED5 is 5'-CCG CGG CCA GGC TAG CTA CAA CGA CCT GGA CGA T-3' (3' T is inverted; catalytic domain is underlined). **d**, DNAzyme cleavage of 206 nt in vitro transcript. The schematic shows the NGFI-A 206 nt in vitro transcript and expected cleavage products (163 and 43 nts). Arrow indicates the expected site of cleavage. Data in each panel of this figure is representative of 2 or more independent experiments.

Figure 2 NGFI-A DNAzyme inhibits the induction of NGFI-A mRNA and protein by serum. **a**, Northern blot analysis was performed with 25 μg of total RNA. The blot was stripped and reprobed for β-Actin. Autoradiograms were analyzed by scanning densitometry and the ordinate axis is expressed as NGFI-A band intensity as a fraction of β-Actin band intensity. The mean and standard errors of the mean are indicated in the figure. Data is representative of 2 independent experiments. * indicates $P<0.05$ (Student's paired t-test) as compared to control (FBS alone). **b**, Western blot analysis was performed using antibodies to Egr-1/NGFI-A, Sp1 or c-Fos. The Coomassie Blue stained gel demonstrates that uniform amounts of protein were loaded per lane. The sequence of EDC is 5'-CGC CAT TAG GCT AGC TAC AAC GAC CTA GTG AT-3'; 3' T is inverted; catalytic domain is underlined). SFM denotes serum-free medium.

Figure 3 SMC proliferation is inhibited by NGFI-A DNAzyme. **a**, Assessment of total cell numbers by Coulter counter. Growth-arrested SMCs that had been exposed to serum and/or DNAzyme for 3 days were trypsinized followed by quantitation of the suspension. The sequence of AS2 is 5'-CTT GGC CGC TGC CAT-3'. **b**, Proportion of cells incorporating Trypan Blue after exposure to serum and/or DNAzyme. Cells were stained incubated in 0.2%

(w:v) Trypan Blue at 22 °C for 5 min prior to quantitation by hemocytometer in a blind manner. **c**, Effect of ED5 on pup SMC proliferation. Growth-arrested WKY12-22 cells exposed to serum and/or DNAzyme for 3 days were resuspended and numbers were quantitated by Coulter counter. Data is representative of 2 independent experiments performed in triplicate. The mean and standard errors of the mean are indicated in the figure. * indicates $P<0.05$ (Student's paired t-test) as compared to control (FBS alone).

Figure 4 Cellular localization and stability of NGFI-A DNAzymes. **a**, ED5 and ED5SCR localize predominantly within SMC nuclei. Growth-arrested SMC were transfected with FITC-(5' end)-labeled DNAzyme and fluorescence microscopy was performed after 24 h at 37 °C. ODN denotes oligonucleotide. Magnification 400x. **b**, 3' inverted T confers resistance to nucleolytic degradation in serum. ^{32}P-ED5 or ^{32}P-ED5SCR bearing a 3'-T in the correct or inverted position was incubated in 5% FBS or SFM at 37 °C for the times indicated prior to electrophoresis on 12% denaturing polyacrylamide gels and subsequent autoradiography.

Figure 5 NGFI-A DNAzyme inhibits SMC repair after mechanical injury. Hematoxylin-eosin stained SMC cultures **A**, immediately after scraping, **B**, 3 days after injury, or 3 days after injury in the presence of **C**, ED5 or **D**, its scrambled counterpart, ED5SCR. Magnification 100x. Data is representative of 3 independent experiments.

Figure 6 NGFI-A DNAzyme inhibition of neointima formation in the rat carotid artery. A neointima was achieved 18 days after permanent ligation of the right common carotid artery. DNAzyme (500 μg) or vehicle alone was applied adventitially at the time of ligation and again after 3 days. **a**, Sequence-specific inhibition of neointima formation. Neointimal and medial areas of 5 consecutive sections per rat (5 rats per group) taken at 250 μm intervals from the point of ligation were determined digitally and expressed as a ratio per group. The mean and standard errors of the mean are indicated by the ordinate axis. * denotes $P<0.05$ as compared to the Lig, Lig+Veh or Lig+Veh+ED5SCR groups using the Wilcoxen rank sum test for unpaired data. Lig denotes ligation, Veh denotes vehicle. **b**, Representative cross-sections of carotid arteries 18 d after **A**, ligation alone, **B**, ligation with

adventitial application of vehicle, or vehicle containing **C**, ED5 or **D**, ED5SCR. Sections (5 μm) were stained with hematoxylin and eosin. Magnification 250x. *N* denotes neointima, *M* denotes media, *A* denotes adventitia.

Figure 7 Human EGR-1 DNAzyme cleaves EGR-1 RNA in both a dose-dependent (upper panel) and time-dependent (lower panel) manner. Sequence of hED5SCR is 5'-GCC AGC CGC GGC TAG CTA CAA CGA AGG TGC CAC T-3' (3' T is inverted; catalytic domain is underlined). Sequence of hED5 appears in the legend of Fig. 1 and that of the substrate appears in the legend to Table 2.

DETAILED DESCRIPTION OF THE INVENTION

Materials and Methods

ODN synthesis. DNazymes were synthesized commercially (Oligos Etc., Inc.) with an inverted T at the 3' position unless otherwise indicated. Substrates in cleavage reactions were synthesized with no such modification. Where indicated ODNs were 5'-end labeled with γ^{32}P-dATP and T4 polynucleotide kinase (New England Biolabs). Unincorporated label was separated from radiolabeled species by centrifugation on Chromaspin-10 columns (Clontech).

In vitro transcript and cleavage experiments. A ^{32}P-labelled 206 nt NGFI-A RNA transcript was prepared by in vitro transcription (T3 polymerase) of plasmid construct pJDM8 (as described in Milbrandt, 1987, the entire contents of which are incorporated herein by reference) previously cut with *Bgl* II. Reactions were performed in a total volume of 20 μl containing 10 mM $MgCl_2$, 5 mM Tris pH 7.5, 150 mM NaCl, 4.8 pmol of in vitro transcribed or synthetic RNA substrate and 60 pmol DNAzyme (1:12.5 substrate to DNAzyme ratio), unless otherwise indicated. Reactions were allowed to proceed at 37 °C for the times indicated and quenched by transferring an aliquot to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were run on 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

Culture conditions and DNAzyme transfection. Primary rat aortic SMCs were obtained from Cell Applications, Inc., and grown in Waymouth's medium, pH 7.4, containing 10% fetal bovine serum (FBS), 50 μg/ml streptomycin and 50 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. SMCs were used in experiments between passages 3-7. Pup rat SMCs (WKY12-22 (as described in Lemire et al, 1994, the entire contents of which are incorporated herein by reference)) were grown under similar conditions. Subconfluent (60-70%) SMCs were incubated in serum-free medium (SFM) for 6 h prior to DNAzyme (or antisense ODN, where indicated) transfection (0.1 μM) using Superfect in accordance with manufacturer's instructions (Qiagen). After 18 h, the cells were washed with phosphate-buffered saline (PBS), pH 7.4 prior to transfection a second time in 5% FBS.

Northern blot analysis. Total RNA was isolated using the TRIzol reagent (Life Technologies) and 25 μg was resolved by electrophoresis prior to transfer to Hybond-N+ membranes (NEN-DuPont). Prehybridization, hybridization with α^{32}P-dCTP-labeled Egr-1 or β-Actin cDNA, and washing was performed essentially as previously described (Khachigian et al, 1995).

Western blot analysis. Growth-quiescent SMCs in 100 mm plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, and incubated with 5% FBS for 1 h. The cells were washed in cold PBS, pH 7.4, and extracted in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasylol, 10 μg/ml leupeptin, 1% aprotinin and 2 mM PMSF. Twenty four μg protein samples were loaded onto 10% denaturing SDS-polyacrylamide gels and electroblotted onto PVDF nylon membranes (NEN-DuPont). Membranes were air dried prior to blocking with non-fat skim milk powder in PBS containing 0.05% (w:v) Tween 20. Membranes were incubated with rabbit antibodies to Egr-1 or Sp1 (Santa Cruz Biotechnology, Inc.) (1:1000) then with HRP-linked mouse anti-rabbit Ig secondary antiserum (1:2000). Where mouse monoclonal c-Fos (Santa Cruz Biotechnology, Inc.) was used, detection was achieved with HRP-linked rabbit anti-mouse Ig. Proteins were visualized by chemiluminescent detection (NEN-DuPont).

Assays of cell proliferation. Growth-quiescent SMCs in 96-well titer plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, then exposed to 5% FBS at 37 °C for 72 h. The cells were rinsed with PBS, pH 7.4, trypsinized and the suspension was quantitated using an automated Coulter counter.

Assessment of DNAzyme stability. DNAzymes were 5'-end labeled with γ^{32}P-dATP and separated from free label by centrifugation. Radiolabeled DNAzymes were incubated in 5% FBS or serum-free medium at 37 °C for the times indicated. Aliquots of the reaction were quenched by transfer to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were applied to 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

SMC wounding assay. Confluent growth-quiescent SMCs in chamber slides (Nunc-InterMed) were exposed to ED5 or ED5SCR for 18 h prior to a single scrape with a sterile toothpick. Cells were treated with mitomycin C (Sigma) (20 μM) for 2 h prior to injury (Pitsch et al, 1996; Horodyski & Powell, 1996). Seventy-two h after injury, the cells were washed with PBS, pH 7.4, fixed with formaldehyde then stained with hematoxylin-eosin.

Rat arterial ligation model and analysis. Adult male Sprague Dawley rats weighing 300-350 g were anaesthetised using ketamine (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The right common carotid artery was exposed up to the carotid bifurcation via a midline neck incision. Size 6/0 non-absorbable suture was tied around the common carotid proximal to the bifurcation, ensuring cessation of blood flow distally. A 200 μl solution at 4 °C containing 500 μg of DNAzyme (in DEPC-treated H_2O), 30 μl of transfecting agent and Pluronic gel P127 (BASF) was applied around the vessel in each group of 5 rats, extending proximally from the ligature for 12-15 mm. These agents did not inhibit the solidification of the gel at 37 °C. After 3 days, vehicle with or without 500 μg of DNazyme was administered a second time. Animals were sacrificed 18 days after ligation by lethal injection of phenobarbitone, and perfusion fixed using 10% (v:v) formaldehyde perfused at 120 mm Hg. Both carotids were then dissected free and placed in 10%

formaldehyde, cut in 2 mm lengths and embedded in 3% (w:v) agarose prior to fixation in paraffin. Five μm sections were prepared at 250 μm intervals along the vessel from the point of ligation and stained with hematoxylin and eosin. The neointimal and medial areas of 5 consecutive sections per rat were determined digitally using a customized software package (Magellan) (Halasz & Martin, 1984) and expressed as a mean ratio per group of 5 rats.

Results and Discussion

The 7x7 nt arms flanking the 15 nt DNAzyme catalytic domain in the original DNAzyme design 7 were extended by 2 nts per arm for improved specificity (L.-Q. Sun, data not shown) (Fig. 1a). The 3' terminus of the molecule was capped with an inverted 3'-3'-linked thymidine (T) (Fig. 1a) to confer resistance to 3'->5' exonuclease digestion. The sequence in both arms of ED5 was scrambled (SCR) without altering the catalytic domain to produce DNAzyme ED5SCR (Fig. 1a).

A synthetic RNA substrate comprised of 23 nts, matching nts 805 to 827 of NGFI-A mRNA (Fig. 1a) was used to determine whether ED5 had the capacity to cleave target RNA. ED5 cleaved the ^{32}P-5'-end labeled 23-mer within 10 min (Fig. 1b). The 12-mer product (Fig. 1b) corresponds to the length between the A(816)-U(817) junction and the 5' end of the substrate (Fig. 1a). In contrast, ED5SCR had no demonstrable effect on this synthetic substrate (Fig. 1b). Specific ED5 catalysis was further demonstrated by the inability of the human equivalent of this DNAzyme (hED5) to cleave the rat substrate over a wide range of stoichiometric ratios (Fig. 1c). Similar results were obtained using ED5SCR (data not shown). hED5 differs from the rat ED5 sequence by 3 of 18 nts in its hybridizing arms (Fig. 1c & Table 2). The catalytic effect of ED5 on a ^{32}P-labeled 206 nt fragment of native NGFI-A mRNA prepared by *in vitro* transcription was then determined. The cleavage reaction produced two radiolabeled species of 163 and 43 nt length (Fig. 1d) consistent with DNAzyme cleavage at the A(816)-U(817) junction (Fig. 1d). In other experiments, ED5 also cleaved a ^{32}P-labeled NGFI-A transcript of 1960 nt length in a specific and time-dependent manner (data not shown).

Table 2. DNAzyme target sites in mRNA.

Similarity between the 18 nt arms of ED5 or hED5 and the mRNA of rat NGFI-A or human EGR-1 (among other transcription factors) is expressed as a percentage. The target sequence of ED5 in NGFI-A mRNA is 5'-807-A CGU CCG GGA UGG CAG CGG-825-3' (rat NGFI-A sequence), and that of hED5 in EGR-1 is 5'-262-U CGU CCA GGA UGG CCG CGG-280-3' (Human EGR-1 sequence). Nucleotides in bold indicate mismatches between rat and human sequences. Data obtained by a gap best fit search in ANGIS using sequences derived from Genbank and EMBL. Rat sequences for Sp1 and c-Fos have not been reported.

Gene	Accession number	Best homology over 18 nts (%)	
		ED5	hED5
Rat NGFI-A	M18416	**100**	84.2
Human EGR-1	X52541	84.2	**100**
Murine Sp1	AF022363	66.7	66.7
Human c-Fos	K00650	66.7	66.7
Murine c-Fos	X06769	61.1	66.7
Human Sp1	AF044026	38.9	28.9

To determine the effect of the DNAzymes on endogenous levels of NGFI-A mRNA, growth-quiescent SMCs were exposed to ED5 prior to stimulation with serum. Northern blot and densitometric analysis revealed that ED5 (0.1 μM) inhibited serum-inducible steady-state NGFI-A mRNA levels by 55% (Fig. 2a), whereas ED5SCR had no effect (Fig. 2a). The capacity of ED5 to inhibit NGFI-A synthesis at the level of protein was assessed by Western blot analysis. Serum-induction of NGFI-A protein was suppressed by ED5 (Fig. 2b). In contrast, neither ED5SCR nor EDC, a DNAzyme bearing an identical catalytic domain as ED5 and ED5SCR but flanked by nonsense arms had any influence on the induction of NGFI-A (Fig.

2b). ED5 failed to affect levels of the constitutively expressed, structurally - related zinc-finger protein, Sp1 (Fig. 2b). It was also unable to block serum-induction of the immediate-early gene product, c-Fos (Fig. 2b) whose induction, like NGFI-A, is dependent upon serum response elements in its promoter and phosphorylation mediated by extracellular-signal regulated kinase (Treisman, 1990, 1994 and 1995; Gashler & Sukhatme, 1995). These findings, taken together, demonstrate the capacity of ED5 to inhibit production of NGFI-A mRNA and protein in a gene-specific and sequence-specific manner, consistent with the lack of significant homology between its target site in NGFI-A mRNA and other mRNA (Table 2).

The effect of ED5 on SMC replication were then determined. Growth-quiescent SMCs were incubated with DNAzyme prior to exposure to serum and the assessment of cell numbers after 3 days. ED5 (0.1 μM) inhibited SMC proliferation stimulated by serum by 70% (Fig. 3a). In contrast, ED5SCR failed to influence SMC growth (Fig. 3a). AS2, an antisense NGFI-A ODN able to inhibit SMC growth at 1 μM failed to inhibit proliferation at the lower concentration (Fig. 3a). Additional experiments revealed that ED5 also blocked serum-inducible ^{3}H-thymidine incorporation into DNA (data not shown). ED5 inhibition was not a consequence of cell death since no change in morphology was observed, and the proportion of cells incorporating Trypan Blue in the presence of serum was not influenced by either DNAzyme (Fig. 3b).

Cultured SMCs derived from the aortae of 2 week-old rats (WKY12-22) are morphologically and phenotypically similar to SMCs derived from the neointima of balloon-injured rat arteries (Seifert et al, 1984; Majesky et al, 1992). The epitheloid appearance of both WKY12-22 cells and neointimal cells contrasts with the elongated, bipolar nature of SMCs derived from normal quiescent media (Majesky et al, 1988). WKY12-22 cells grow more rapidly than medial SMCs and overexpress a large number of growth-regulatory molecules (Lemire et al, 1994), such as NGFI-A (Rafty & Khachigian, 1998), consistent with a "synthetic" phenotype (Majesky et al, 1992; Campbell & Campbell, 1985). ED5 attenuated serum-inducible WKY12-22 proliferation by approximately 75% (Fig. 3c). ED5SCR had no inhibitory effect; surprisingly, it appeared to stimulate growth (Fig. 3c). Trypan Blue exclusion revealed that DNAzyme inhibition was not a consequence of cytotoxicity (data not shown).

To ensure that differences in the biological effects of ED5 and ED5SCR were not the consequence of dissimilar intracellular localization, both DNAzymes were 5'-end labeled with fluorescein isothiocyanate (FITC) and incubated with SMCs. Fluorescence microscopy revealed that both FITC-ED5 (Fig. 4a, center panel) and FITC-ED5SCR (Fig. 4a, lower panel) localized mainly within the nuclei. Punctate fluorescence in this cellular compartment was independent of DNAzyme sequence (Fig. 4a). Fluorescence was also observed in the cytoplasm, albeit with less intensity (Fig. 4a). Cultures not been exposed to DNAzyme showed no evidence of autofluorescence (Fig. 4a, upper panel).

Both molecules were 5'-end labeled with γ^{32}P-dATP and incubated in culture medium to ascertain whether cellular responsiveness to ED5 and ED5SCR was a consequence of differences in DNAzyme stability. Both ^{32}P-ED5 and ^{32}P-ED5SCR remained intact even after 48 h (Fig. 4b). In contrast to ^{32}P-ED5 bearing the 3' inverted T, degradation of ^{32}P-ED5 bearing its 3' T in the correct orientation was observed as early as 1 h (Fig. 4b). Exposure to serum-free medium did not result in degradation of the molecule even after 48 h (Fig. 4b). These findings indicate that inverse orientation of the 3' base in the DNAzyme protects the molecule from nucleolytic cleavage by components in serum.

Physical trauma imparted to SMCs in culture results in outward migration from the wound edge and proliferation in the denuded zone. We determined whether ED5 could modulate this response to injury by exposing growth-quiescent SMCs to either DNAzyme and Mitomycin C, an inhibitor of proliferation (Pitsch et al, 1996; Horodyski & Powell, 1996) prior to scraping. Cultures in which DNAzyme was absent repopulated the entire denuded zone within 3 days (Fig. 5, compare B to A). ED5 inhibited this reparative response to injury (Fig. 5, compare C to B) and prevented additional growth in this area even after 6 days (data not shown). That ED5SCR had no effect in this system (Fig. 5, compare D to B and C) further demonstrates sequence-specific inhibition by ED5.

The effect of ED5 on neointima formation was investigated in a rat model. Complete ligation of the right common carotid artery proximal to the bifurcation results in migration of SMCs from the media to the intima where proliferation eventually leads to the formation of a neointima (Kumar & Lindner, 1997; Bhawan et al, 1977; Buck, 1961). Intimal thickening 18 days

after ligation was inhibited 50% by ED5 (Fig. 6). In contrast, neither its scrambled counterpart (Fig. 6) nor the vehicle control (Fig. 6) had any effect on neointima formation. These findings demonstrate the capacity of ED5 to suppress SMC accumulation in the vascular lumen in a specific manner, and argue against inhibition as a mere consequence of a "mass effect" (Kitze et al, 1998; Tharlow et al, 1996).

Further experiments revealed the capacity of hED5 to cleave (human) EGR-1 RNA. hED5 cleaved its substrate in a dose-dependent manner over a wide range of stoichiometric ratios (Fig. 7). hED5 also cleaved in a time-dependent manner (Fig. 7), whereas hED5SCR, its scrambled counterpart, had no such catalytic property (Fig. 7).

The specific, growth-inhibitory properties of ED5 reported herein suggest that DNAzymes may be useful as therapeutic tools in the treatment of vascular disorders involving inappropriate SMC growth.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this eleventh day of January 1999

UNISEARCH LIMITED
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F B RICE & CO

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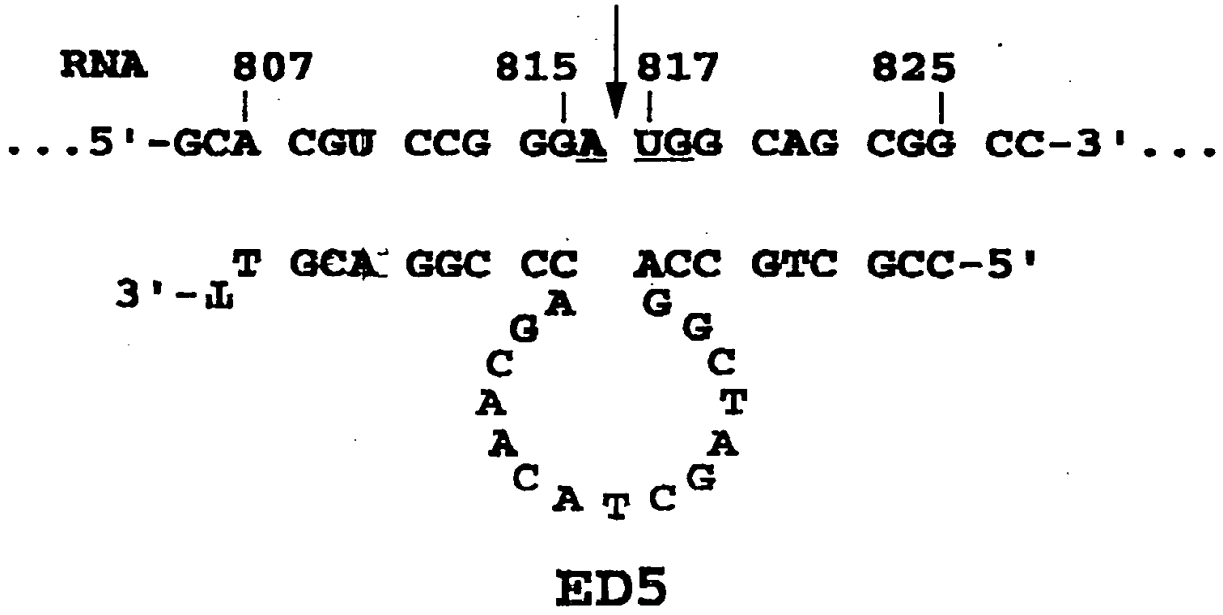
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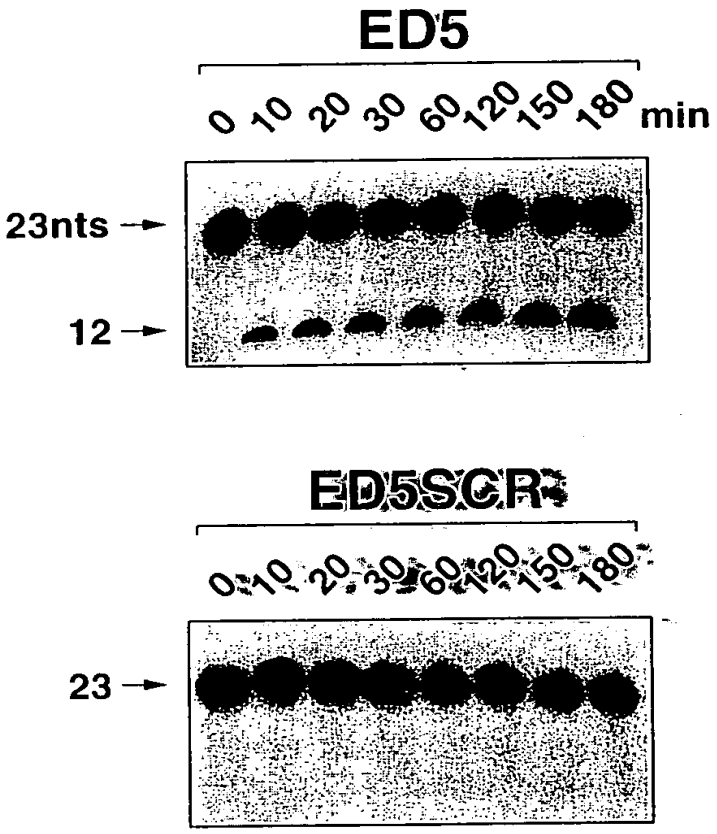
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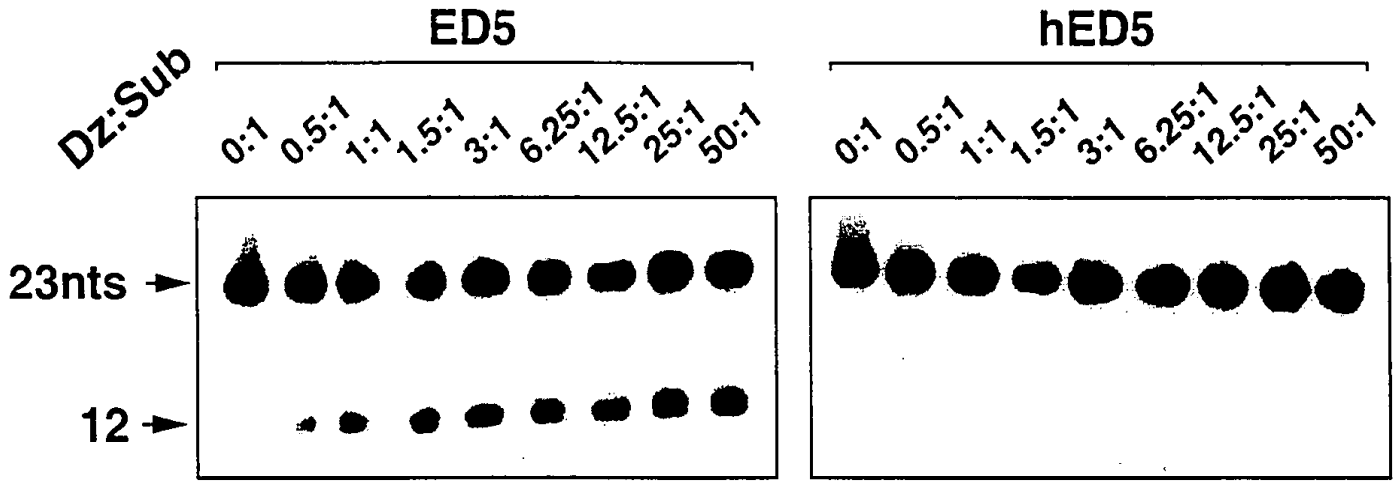

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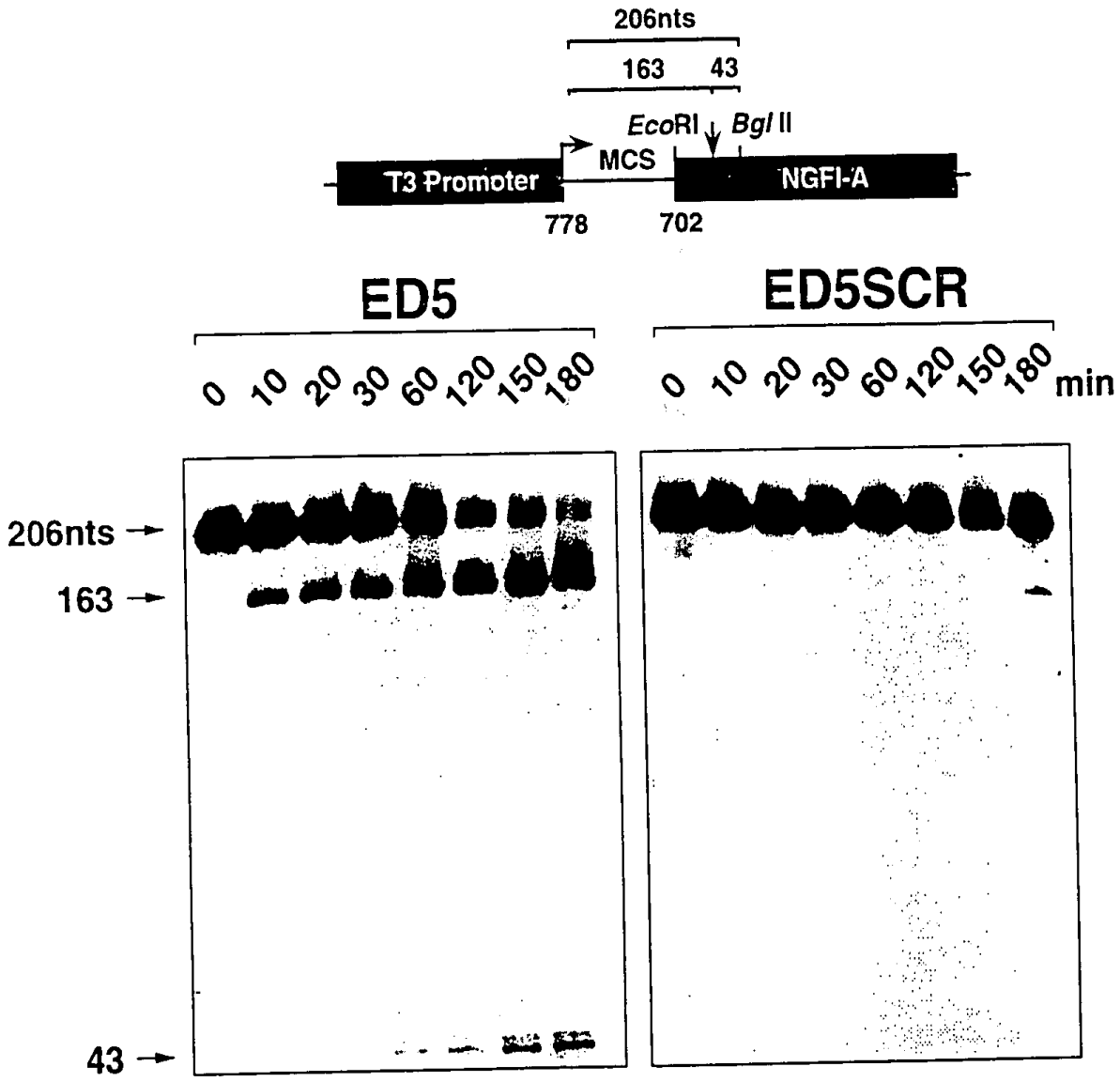
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43

Figure 1D

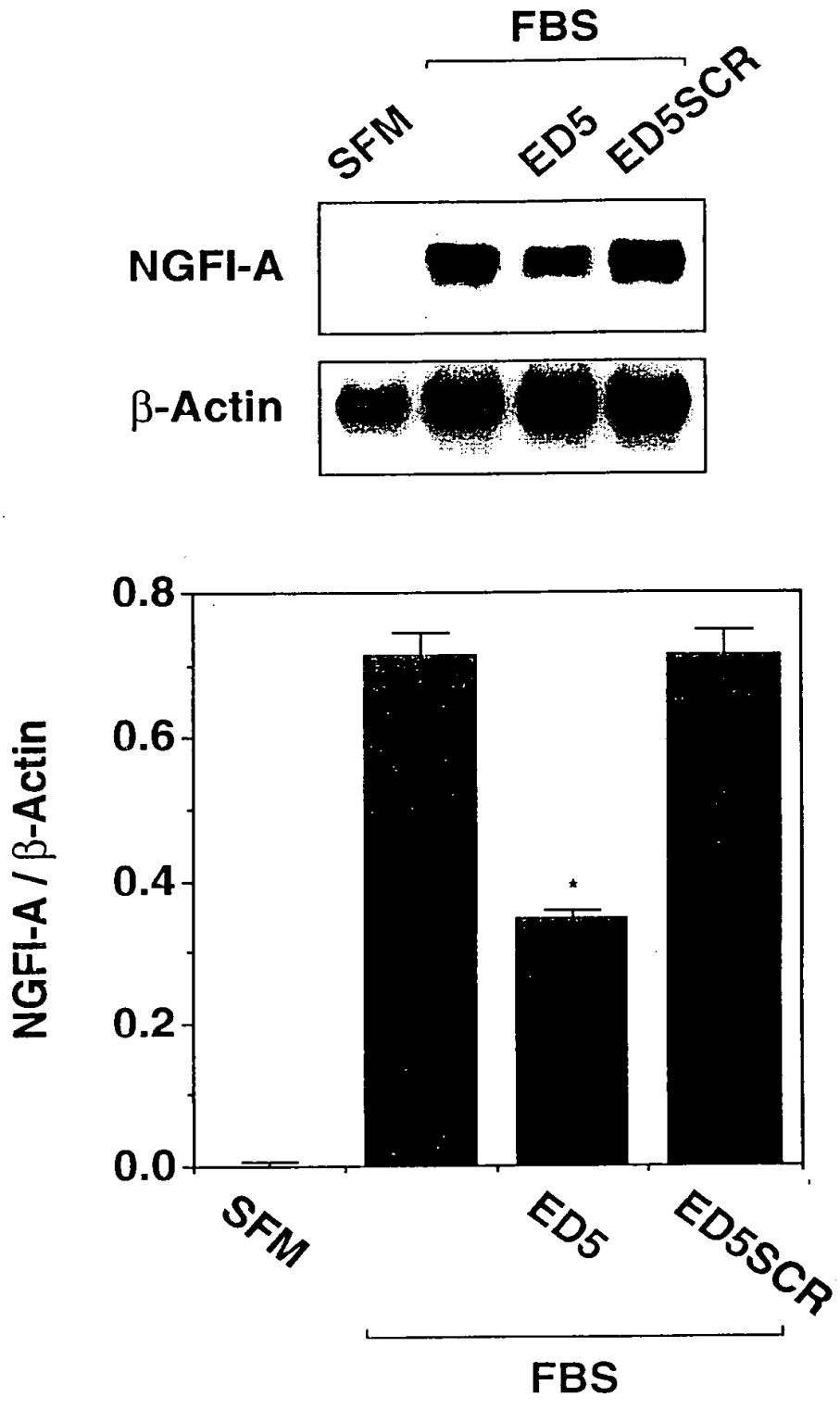
FBS
SFM
ED5
ED5SCR
NGFI-A
β-Actin
NGFI-A / β-Actin
0.8
0.6
0.4
0.2
0.0
*
SFM
ED5
ED5SCR
FBS

Figure 2A

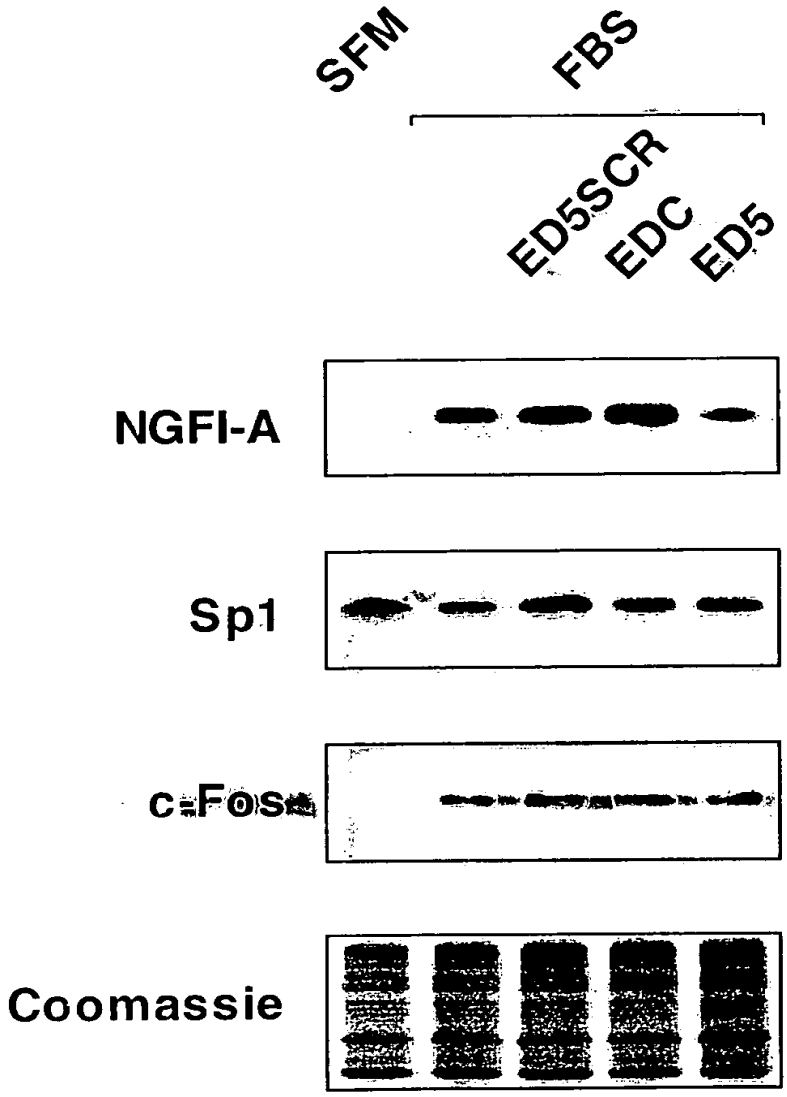

Figure 2B

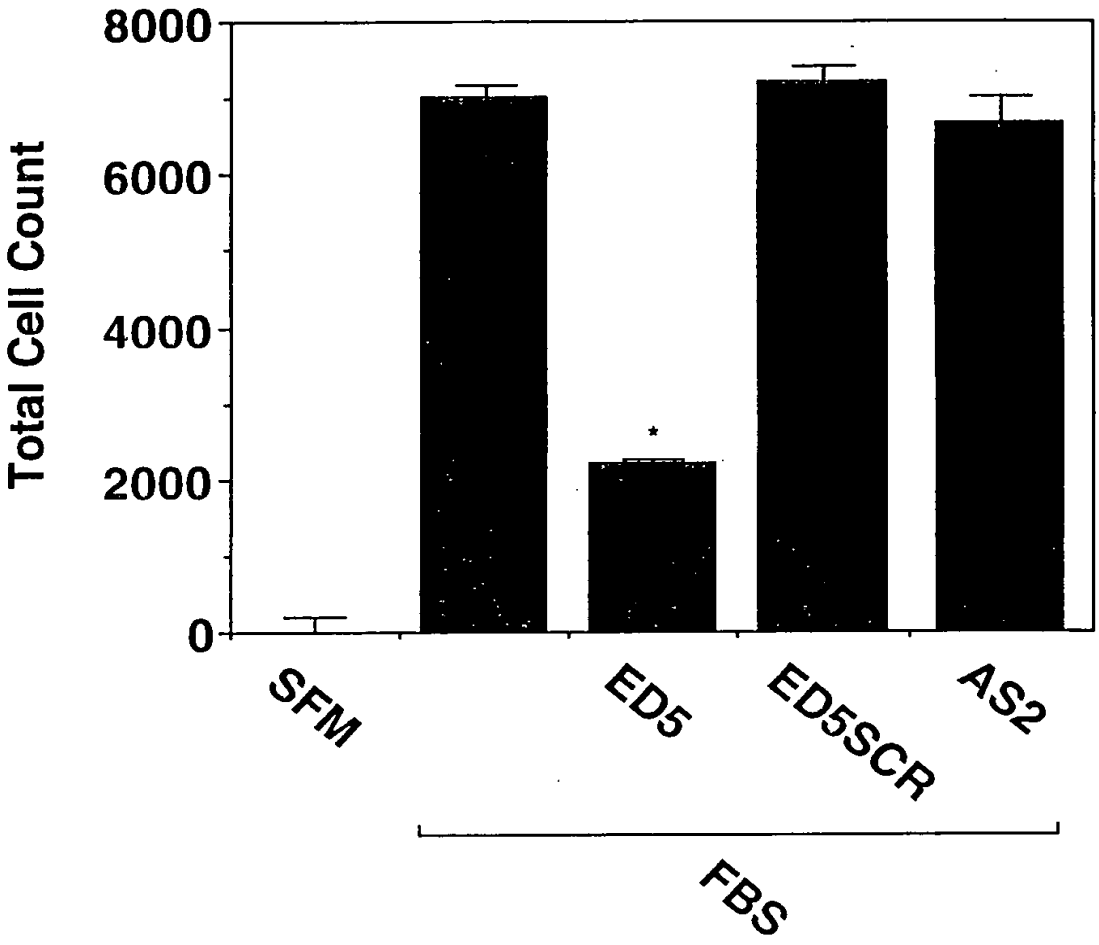

Figure 3A

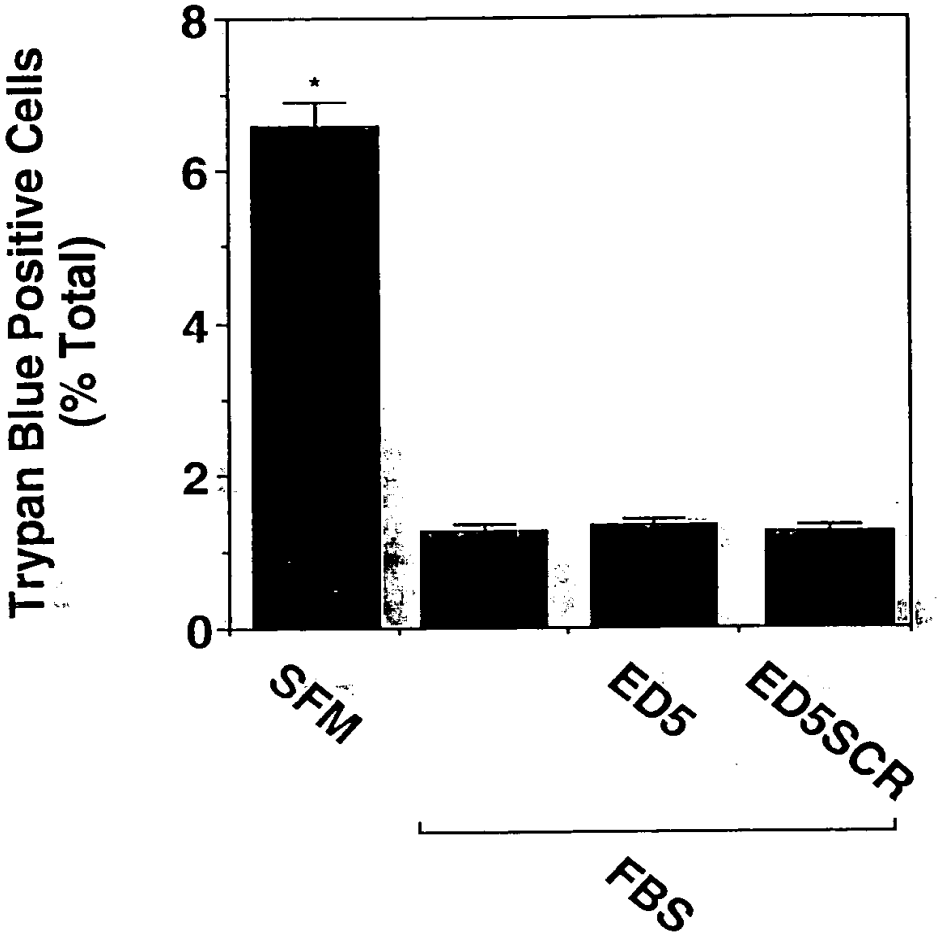

Figure 3B

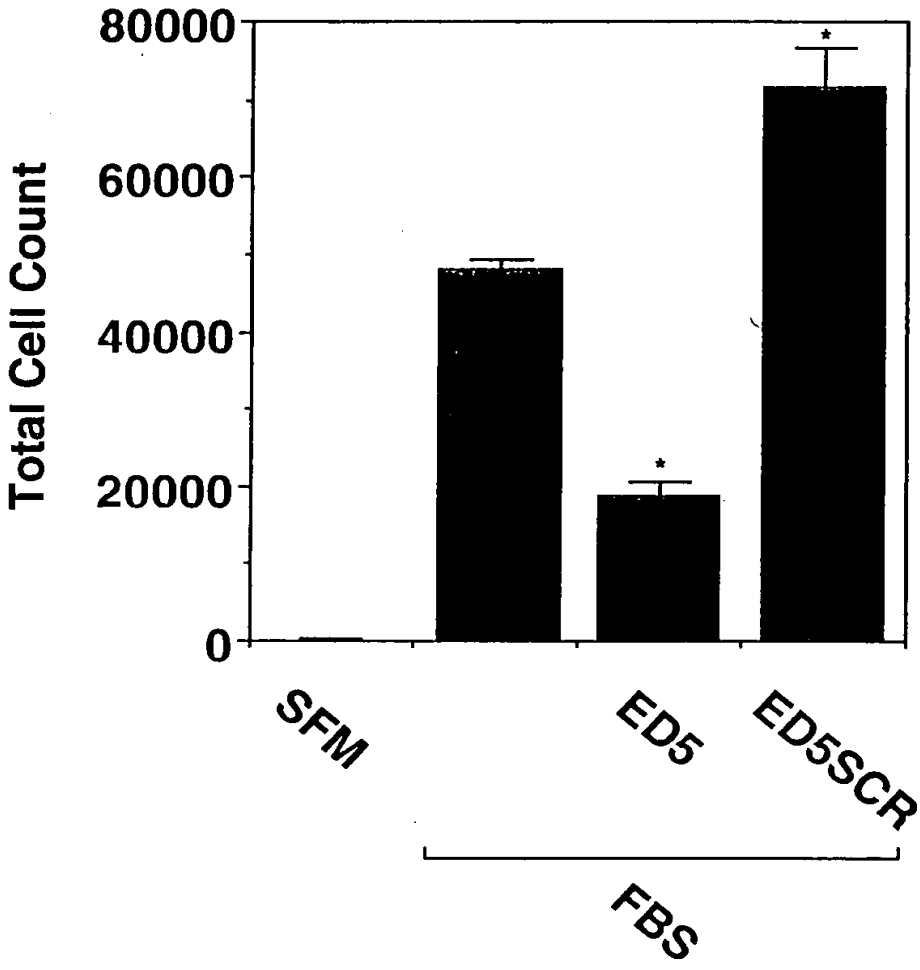
80000
60000
40000
20000
0
Total Cell Count
*
*
SFM
ED5
ED5SCR
FBS

Figure 3C

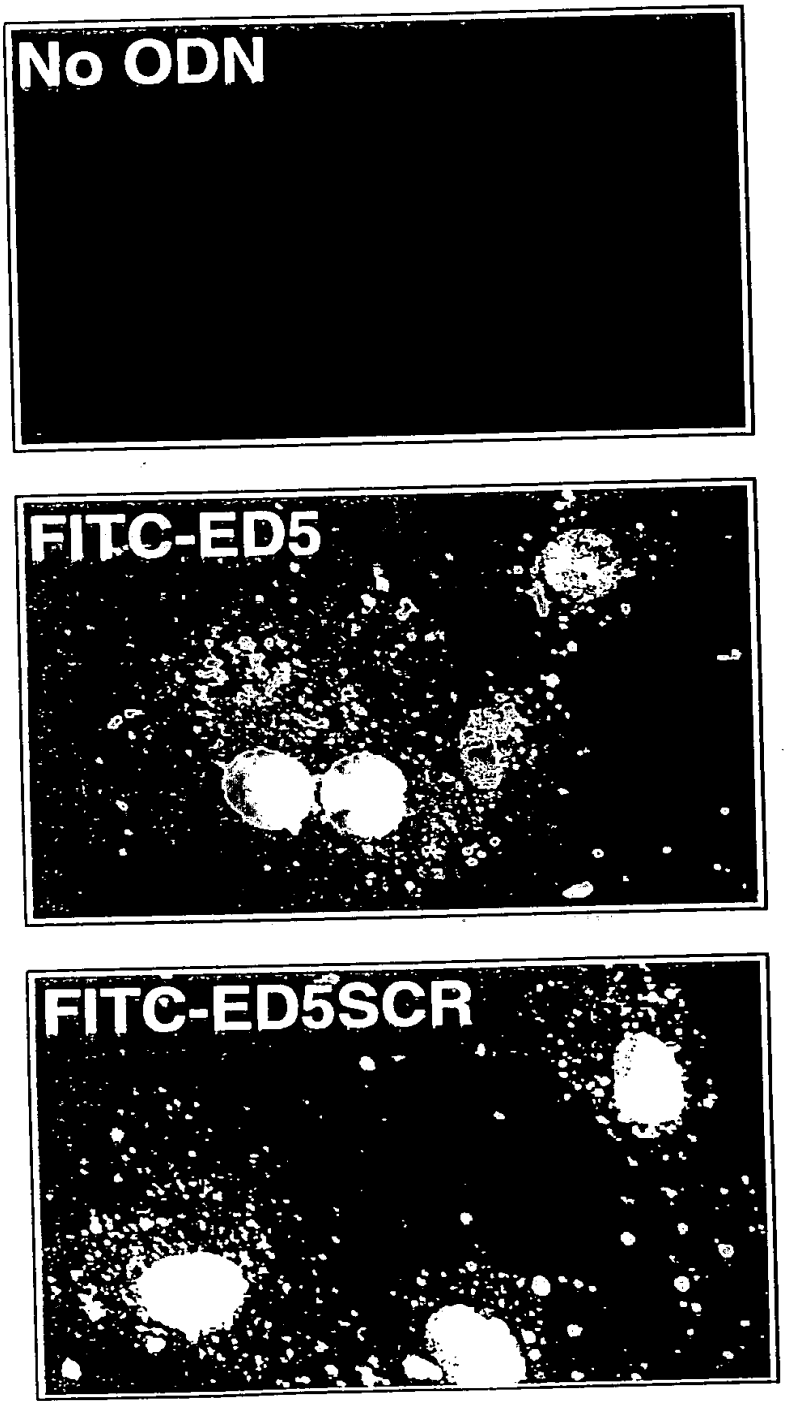

Figure 1A

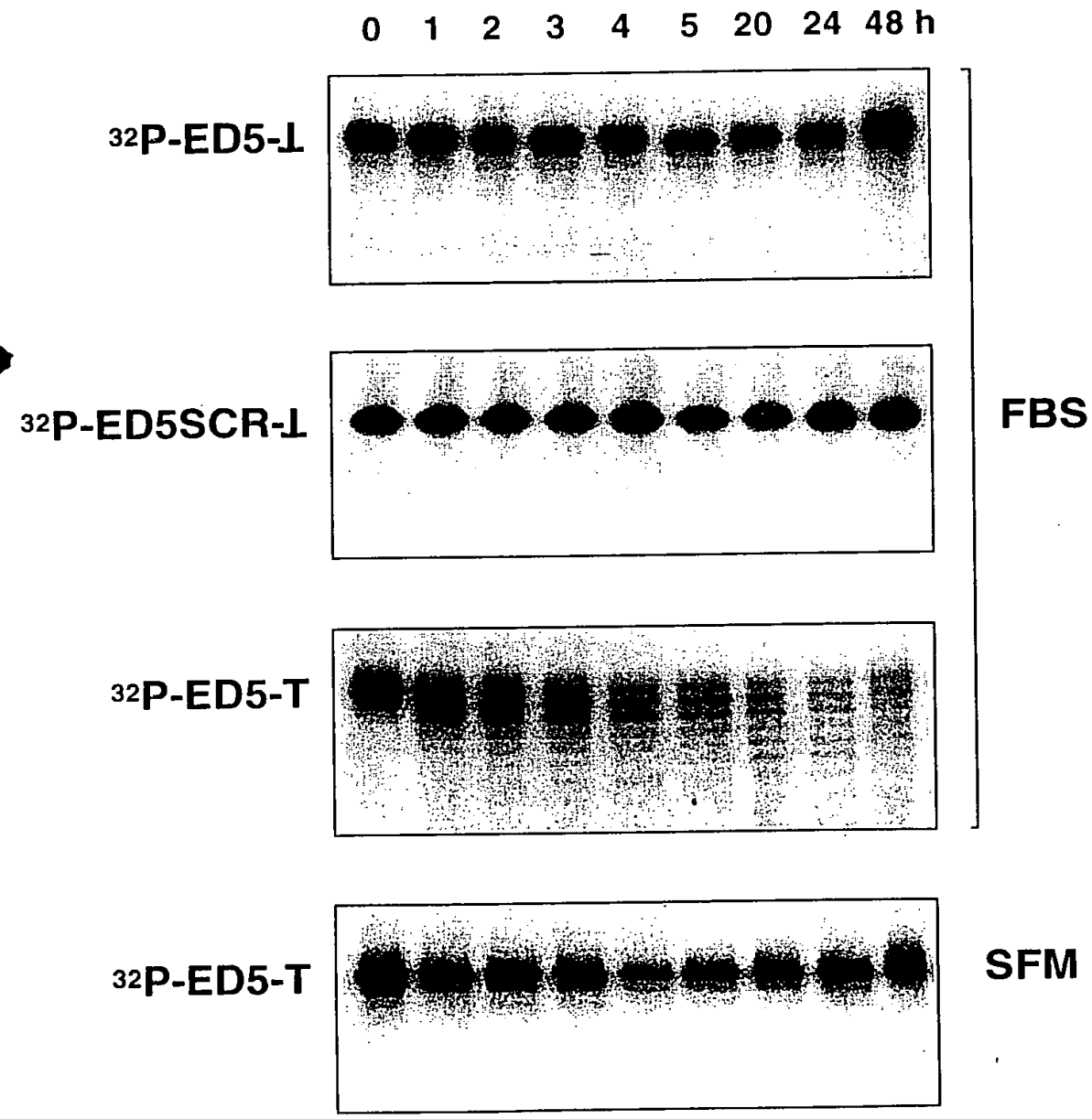

Figure 4B

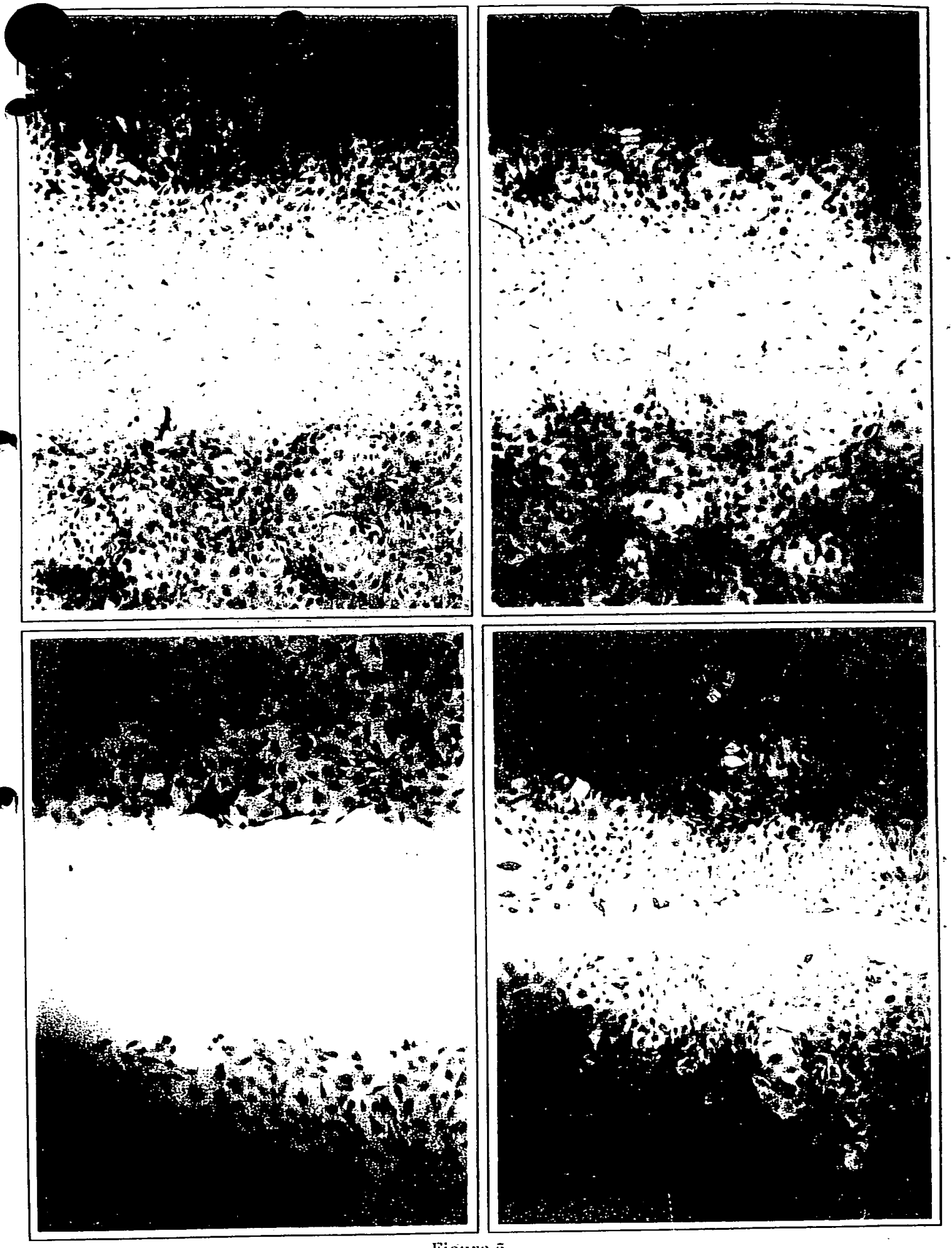

Figure 5

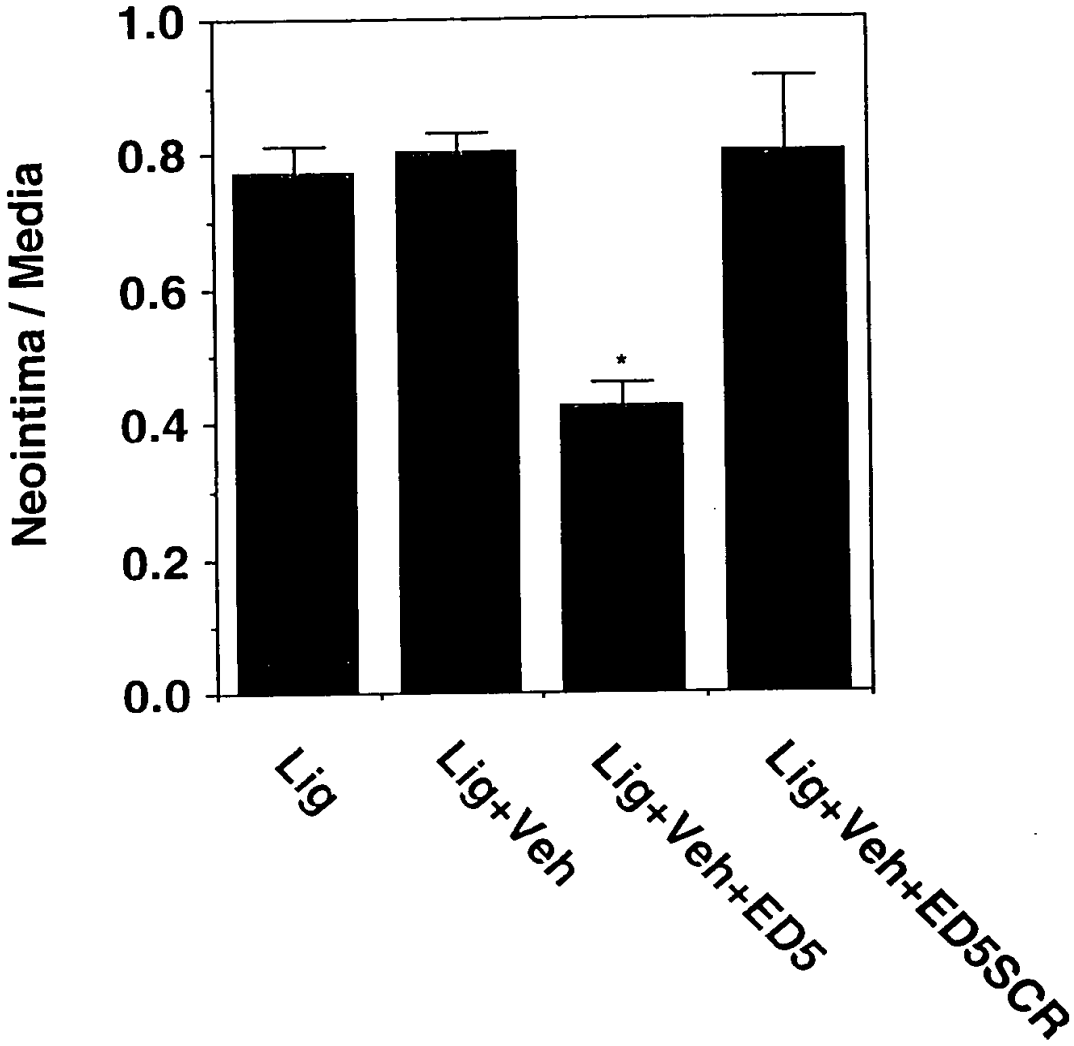
Neointima / Media
1.0
0.8
0.6
0.4
0.2
0.0
*
Lig
Lig+Veh
Lig+Veh+ED5
Lig+Veh+ED5SCR

Figure 6A

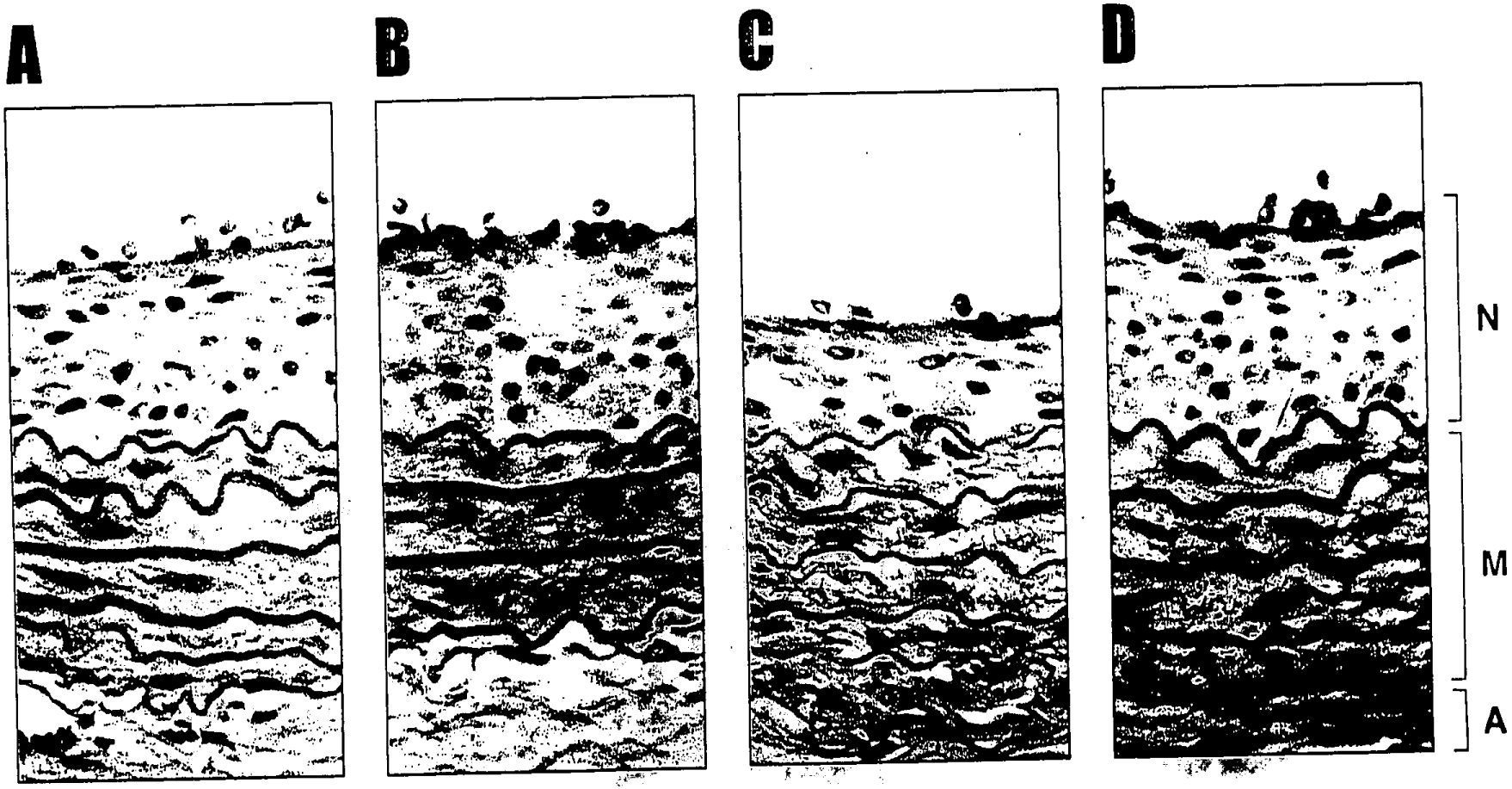

Figure 6B

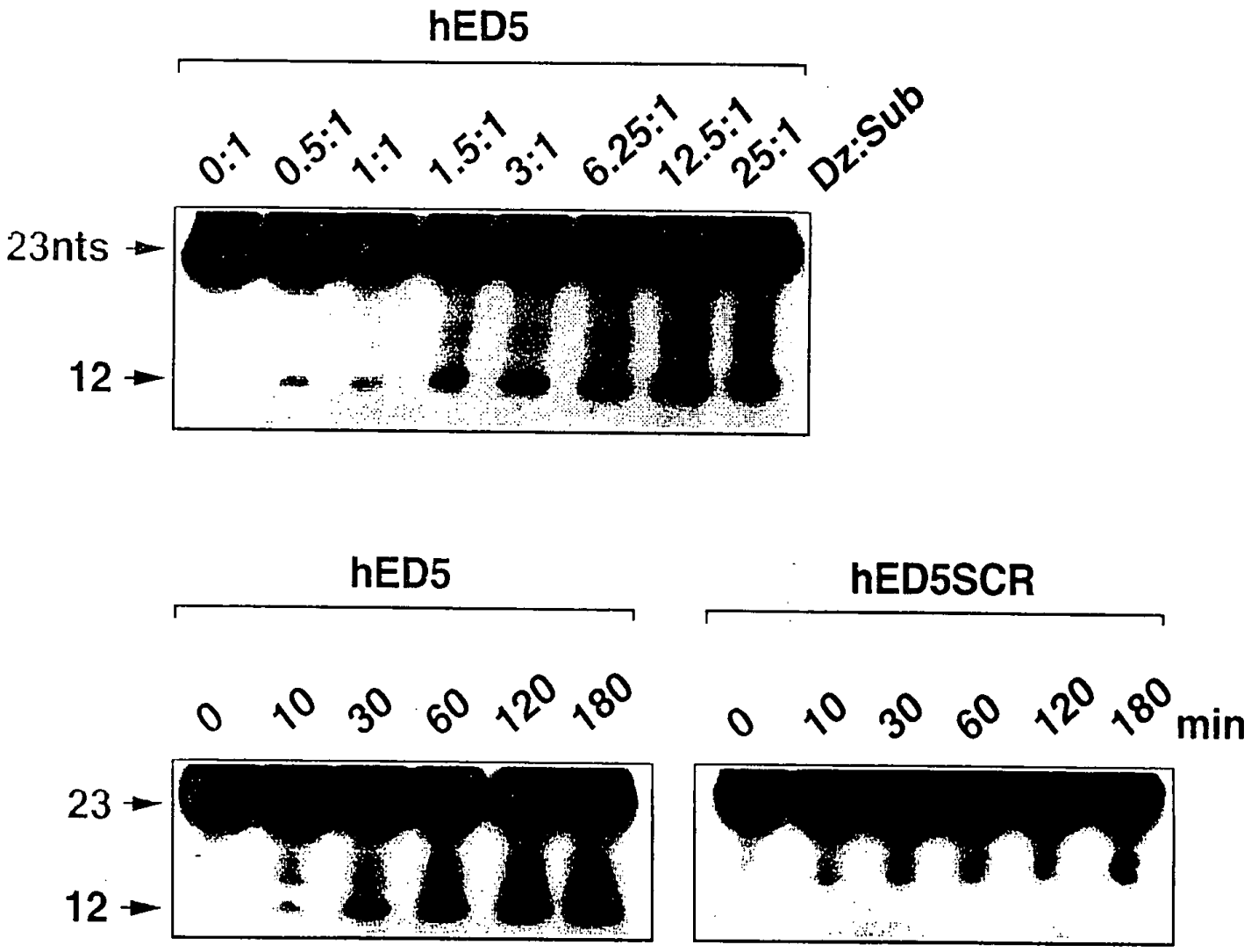
hED5
0:1
0.5:1
1:1
1.5:1
3:1
6.25:1
12.5:1
25:1
Dz:Sub
23nts
12
hED5
hED5SCR
0
10
30
60
120
180
min
23
12

Figure 7